PUBLICATIONS AND PRESENTATIONS
Publications and Presentations

Publications

a) Manuscripts


b) Book Chapter


c) Abstracts


Publications and Presentations

Presentations

National

a) Conference attended

1. 11th International Symposium on Molecular Medicine held from 20-23rd January 2002 at Vadodara, Gujarat.

2. 9th Asian Pacific Congress of Clinical Biochemistry and 28th Annual Conference of Association of clinical biochemists of India (ACBI) held from 9-14th March, 2002 in New Delhi.

3. First Indian Symposium of the Protein Society held from 18-20th October 2002 at Mumbai.

b) Poster presentation


2. Presented the poster at ‘2DGE profile of Aspergillus fumigatus proteins from aspergillosis patients’ in 10th FAOBMB congress held from 7-11th Dec. 2003 at Bangalore, Karnataka.


4. Presented poster and oral on “Identification of major and minor allergens of aspergillus fumigatus based on the sensitization profile in allergic bronchopulmonary aspergillosis patients” in “33rd Indian Immunology Society” meeting from 28th January to 31st January 2007.

Publications and Presentations

International conference

1. Presented poster on “Differential protein expression profile of *Aspergillus fumigatus* on exposure to amphotericin B” at HUPO 5th Annual World Congress, Long Beach, 2006 held from October 28th to November 1st, 2006 at Long Beach, California. Roche Travel Award from HUPO organizers.


Membership of academic societies

- Member of HUPO organization
- Life membership of Indian Immunology Society
IMMUNOPROTEOMIC ANALYSIS OF SECRETORY PROTEINS OF ASPERGILLUS FUMIGATUS WITH SPECIFIC IGE IMMUNOREACTIVITY

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ABSTRACT
Allergenic/antigenic proteins are known to induce Type I and Type III hypersensitivity reactions leading to allergic bronchopulmonary aspergillosis (ABPA) in immunocompetent host. The common structural features or intrinsic properties of the allergens/antigens leading to allergenicity in a host are not well understood. In the current report, comparative analysis of proteins on two dimensional gel electrophoresis (2D-E) and specific IgE immunoblots of A. fumigatus secretory proteins (V, 2" and 3" week culture filtrate proteins) was carried out. We observed a total of 159 proteins in 1", 2" and 3" week culture filtrates of A. fumigatus. Specific IgE immunoreactivity was observed in 75 proteins with different intensity. Third week culture filtrate showed maximum number of proteins, 142, and specific IgE immunoreactive proteins, 65. MALDI-TOF analysis resulted in putative identification of two allergens as hypothetical protein YBL057c from Saccharomyces cerevisiae and unnamed protein product from Debaryomyces hansenii (similar to IPF14568 of Candida albicans). Identification of a repertoire of specific IgE immunoreactive proteins will facilitate the studies on structure-function relationship of these proteins relevant for diagnosis and pathogenesis.

KEY WORDS
Immunoproteomics, Aspergillus fumigatus

INTRODUCTION
Fewer than 60 species of fungi are consistently associated with human infections (1). Most of the pathogenic fungi e.g. Alternaria, curvularia, Epicoccum etc lead to allergic diseases but few e.g. Candida, Aspergillus, Blastomycoses, Cocidioides and Histoplasma are common fungal agents responsible for primary systemic disease (2). Among these, Aspergillus fumigatus is known to cause various allergic [ABPA (allergic bronchopulmonary aspergillosis), extrinsic allergic alveolitis, allergic fungal sinusitis, allergic asthma] and systemic diseases [CNPA (chronic necrotizing pulmonary aspergillosis), IPA (invasive pulmonary aspergillosis)] depending upon the immune status of the host depicting the complexity of pathogenesis (3-5).

In view of the medical importance of Aspergillus fumigatus, its whole genome has been sequenced in 2003 (www.sanger.uk.ac, www.tigr.org). A. fumigatus genome sequencing data proposed approximately 10,000 genes and 30,000 proteins. A total of 326 Expressed Sequence Tags are reported in A. fumigatus (www.ncbi.nlm.nih.gov) (6). Currently, out of over 2334 entries of Aspergillus protein/protein subunit sequences [Swiss-Prot (452) and TrEMBL(1882)] in the SWISS-PROT/TrEMBL database (http.us.expasy.org), 605 are from Aspergillus fumigatus.

Conventional purification methods and recombinant DNA techniques has led to the identification of few functionally important proteins of A. fumigatus (5, 7-9). Proteomics is a rapid approach to study large number of proteins using 2D-E and mass spectrometry. Information generated from proteomics has increased an understanding of biological regulatory networks and has opened novel scientific avenues such as “Structural proteomics” (10). In a recent approach, uncleaved compact domains after proteolytic fragmentation, identified by electrospray ionization mass spectroscopic (ESI-MS) analysis are re-expressed as potential folding units and subjected to the screening processes necessary to generate
crystals suitable for three-dimensional structure determination (11).

Immunoproteomics approach has led to the identification of immunoreactive proteins of Schigella flexneri 2a and Helicobacter pylori (12, 13). Mycelial proteins of A. fumigatus have already been subjected to immunoproteomics, however, the efforts were limited and resulted in identification of Asp f 1 and enolase (14, 15). It is important to note that most of the reported purified antigenic proteins of A. fumigatus are secretory or culture filtrate proteins (1). Asp f 1, a major allergen/antigen of A. fumigatus was shown to be 1000-fold higher in culture filtrate than in hyphae or spore extract (16).

In the present study, we subjected 1st, 2nd and 3rd week culture filtrate proteins of A. fumigatus to immunoproteomic analysis for the first time. The efforts resulted in a comprehensive catalogue of secretory allergens of A. fumigatus and two of them could be assigned with a putative identity after MALDI-TOF analysis.

MATERIALS AND METHODS

A. Preparation of culture filtrate proteins

A. fumigatus secretes different types of proteins at different intervals. In order to identify a large number of secretory allergens/antigens, proteins secreted at 1st, 2nd and 3rd week were studied using proteomic approach. A. fumigatus (strain 285, isolated from the sputum of an ABPA patient visiting Vallabh Bhai Patel Chest Institute, Delhi, India) was grown in a synthetic broth (L-asparagine medium) for one, two and three weeks at 37°C in a stationary culture (17, 18). The filtrate obtained after separating the mycelia was subjected to 80% (w/v) ammonium sulphate precipitation and dialysed extensively against deionised water. The dialysate was lyophilized to get the protein-enriched antigenic fraction. Protein content was estimated by biochinonic acid method (19).

B. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE)

The electrophoretic separation of proteins was performed essentially as described by O'Farrell et al. (20). Isoelectric focusing was carried out in glass tubes (Bio-Rad, Hercules, USA; inner and outer diameters of 1.5 and 4 mm respectively). The tubes were filled with 4.5% tube gel containing 8M Urea, 2% Triton-X-100, 6% ampholine (pH 3-9.5) (Amersham, Uppsala, Sweden). The length of the gel prepared was 11 cm. A pre-run at 50V and 200V for 15 min. was given. A total of 600µg protein (1st, 2nd and 3rd week culture filtrate proteins from clinical isolate of ABPA patient) and standard pH marker (Sigma, Saint Louis, Missouri, USA) was loaded on first dimensional gel and isoelectric focusing was conducted and ran to 200V for 2h, 500V for 2h and 800V for 16h. After isoelectric focusing tubes were incubated for 30 min. at 4°C in equilibration buffer containing 6M Urea, 129.7 mM DTT, 2% SDS, 20% Glycerol, 1.5M Tris. For separation of proteins in second dimension, tube gel was placed on 12% SDS-PAGE. Proteins were visualized by coomassie brilliant blue R-250 staining (Sigma, Saint Louis, Missouri, USA).

C. Sp IgE immunoblotting

Proteins separated in 2D-E gels from 1st, 2nd and 3rd week culture filtrate (wcf) were transferred onto a nitrocellulose membrane (Schleicher and Schuell, Hahnewstrabe, Dassel). The membranes were blocked for 3h with 3% skimmed milk powder in TBST (Tris buffer saline with 0.05% tween 20 + 0.005% Triton-X-100) at 37°C. Test membranes were incubated overnight with pooled sera of 10 suspected cases of ABPA [with skin prick test positive and high titre of specific IgG antibodies (with ELISA values ranging from 0.740-1.483) and IgE antibodies (with ELISA values ranging from 0.170-0.287)] diluted 1:5 in TBST + 1% BSA + 5mM CaCl₂ at 4°C. Control membranes were incubated with pooled sera of 10 healthy individuals [with skin prick test negative with specific IgG (with ELISA values ranging from 0.030-0.037) and IgE antibodies (with ELISA values ranging from 0.032-0.062)]. All the membranes were further incubated with secondary antibodies (anti-human IgE e-chain specific-horse-raddish peroxidase antibodies from Sigma) diluted 1:1000 in TBST + 1% BSA + 5mM CaCl₂ at 37°C and developed with substrate (3, 3’ Diaminobenzidine).

Comparative analysis of 2D gels and specific IgE immunoblots of 1st, 2nd and 3rd week culture filtrate proteins was carried out using a Microcal origin: version 3.

D. In situ tryptic digestion of proteins

Gel slices were trimmed to approx. 1 mm² and washed 3X in 50% acetonitrile (ACN) for 8.0 (400 ml, 15 min. each time) to destain and soaked in 100% ACN for 5 min. to dehydrate the gels. ACN was removed and gel slices were dried in speed vacum for 20-30 min. Dried gel slices were rehydrated with approx. 10ml cold trypsin solution (Sigma Sequencing Grade, Modified Trypsin 10-15ng/ml in 25mM ammonium bicarbonate pH 8.0). Rehydrated gel slices were incubated at 37°C for 16 hours followed by soaking in 25-50 µl 50%ACN/5%TFA for 30-60 min. with gentle agitation. Supernatant was lyophilized at room temperature and reconstituted by adding 3.0 µl of 50%ACN/0.1%TFA to
the bottom of the tube and gently pipetted to dissolve the extracted peptides.

E. Matrix-assisted laser desorption-ionization time of flight mass spectrometry (MALDI-TOF) analysis
Peptide mass mapping was performed with 0.5 μl reconstituted extract with 0.5 μl fresh α-cyano-4-hydroxycinnamic acid (CHCA, Applied biosystem, Foster City, USA) matrix on a MALDI plate. Cal Mix 2 was spotted adjacent to sample for close external calibration. MALDI spectra were acquired in reflector mode with a method optimized for high resolution in 600-3000 Da range. The samples were analyzed by MALDI-TOF on Ultraflex II, Bruker Daltonics. Peak list obtained was calibrated with internal Trypsin peaks T7 (842.5099) and T4 (2211.1046). Peptide masses were searched in fungi using the Mascot search engine (MSDB database) (www.matrixscience.com) for protein identification. The parameters were set as follows: Mascot score above 53 were taken as the cut off, mass tolerance 10 ppm, molecular mass unrestricted, charge +1 and missed cleavage 1 while performing the search.

RESULTS AND DISCUSSION
Comparative analysis of 2D-E and specific IgE immunoblots (with pooled patient sera) of 1st, 2nd and 3rd week culture filtrate protein fractions of A. fumigatus showed 38, 92 and 142 proteins respectively out of which 16, 49 and 65 proteins showed specific IgE immunoreactivity (Fig. 1,2,3,4, Table 1). We have observed a total of 75 proteins with specific IgE immunoreactivity in culture filtrate whereas information is available only for 27 proteins (25 secretory proteins and 2 intracellular proteins) in the public domain identified from mycelia as well as culture filtrate including 23 recombinant allergens/antigens of A. fumigatus (Table 1). Out of 25 secretory allergens available in literature, we observed 25 proteins with similar molecular mass and pi showing binding to IgE antibodies but their identity needs to be confirmed by MALDI-TOF and ESI-MS/MS analysis. Third week culture filtrate showed maximum number of proteins, 142, and specific IgE immunoreactive proteins, 65. It is already reported that third week culture filtrate proteins are relevant for immunodiagnosis on SDS-PAGE (19).

Figure 1.
2D gel electrophoresis (a) and corresponding specific IgE immunoblots with test sera (b) and control sera (c) of 1st week culture filtrate proteins of A. fumigatus (clinical isolate of ABPA patient). 2D-E (after staining with Coomassie R-250 staining) detected 38 proteins and immunoblots detected 16 with specific IgE immunoreactivity in 1st week culture filtrate. Proteins (600 μg) were separated by isoelectric focusing on tube gel, 11cm, pi 3-10, and then by SDS–PAGE, 12.0%
Figure 2
2D gel electrophoresis (d) and corresponding specific IgE immunoblots with test sera (e) and control sera (f) of 2nd week culture filtrate proteins of *A. fumigatus* (clinical isolate of ABPA patient). 2D-E (after staining with Coomassie R-250 staining) detected 92 proteins and immunoblots detected 49 with specific IgE immunoreactivity in 2nd week culture filtrate. Proteins (600 µg) were separated by isoelectric focusing on tube gel, 11cm, pl 3–10 and then by SDS–PAGE, 12.0%.

Figure 3
2D gel electrophoresis (g) and corresponding specific IgE immunoblots with test sera (h) and control sera (i) of 3rd week culture filtrate proteins of *A. fumigatus* (clinical isolate of ABPA patient). 2D-E (after staining with Coomassie R-250 staining) detected 142 proteins and immunoblots detected 65 with specific IgE immunoreactivity in 3rd week culture filtrate. Two protein spots (spot no. 131 and 147) in 2D-E marked with arrow were subjected to MALDI-TOF analysis. Proteins (600 µg) were separated by isoelectric focusing on tube gel, 11cm, pl 3–10 and then by SDS–PAGE, 12.0%.
Proteins observed specifically in 1\textsuperscript{st}, 2\textsuperscript{nd} and 3\textsuperscript{rd} week are 2, 12 and 63 respectively. Proteins observed in 1\textsuperscript{st} and 2\textsuperscript{nd} week, 2\textsuperscript{nd} and 3\textsuperscript{rd} week and 1\textsuperscript{st} and 3\textsuperscript{rd} week are 3, 46 and 2 respectively. Proteins commonly observed in 1\textsuperscript{st}, 2\textsuperscript{nd} and 3\textsuperscript{rd} week are 31 (Table 2). Proteins showing high immunoreactivity with test sera (patient) were considered as specific IgE immunoreactive proteins (Fig 1, 2, 3). Immunoblot with control sera (healthy) showed negligible binding to culture filtrate proteins. Out of a total of seventy five specific IgE immunoreactive proteins, seven proteins were observed in 1\textsuperscript{st} and/or 2\textsuperscript{nd} week. Among these, five proteins (spot no. 26, 74, 75, 114 and 159) were observed in 2\textsuperscript{nd} week and only one protein (spot no. 123) in 1\textsuperscript{st} week. One protein (spot no. 97) was commonly observed in 1\textsuperscript{st} and 2\textsuperscript{nd} week (Table 1).

2D-E of three week culture filtrate was observed to have proteins with similar molecular mass and different pI's at 13kD
8.5.9.6.3.
9. 78.4, 3.3.
10. 27.2, 3.3.
11. 48.1, 3.3.
12. 57.0, 3.3.
13. 66.9, 3.3.
14. 76.3, 3.3.
15. 86.7, 3.3.
16. 96.1, 3.3.
17. 10.5, 3.3.
18. 20.9, 3.3.
19. 30.3, 3.3.
20. 40.7, 3.3.
21. 50.1, 3.3.
22. 60.5, 3.3.
23. 70.9, 3.3.
24. 81.3, 3.3.
25. 91.7, 3.3.
26. 102.1, 3.3.
27. 112.5, 3.3.
28. 122.9, 3.3.
29. 133.3, 3.3.
30. 143.7, 3.3.
31. 154.1, 3.3.
32. 164.5, 3.3.
33. 174.9, 3.3.
34. 185.3, 3.3.
35. 195.7, 3.3.
36. 206.1, 3.3.
37. 216.5, 3.3.
38. 226.9, 3.3.
39. 237.3, 3.3.
40. 247.7, 3.3.
41. 258.1, 3.3.
42. 268.5, 3.3.
43. 278.9, 3.3.
44. 289.3, 3.3.
45. 299.7, 3.3.
46. 300.2, 3.3.
47. 310.6, 3.3.
48. 321.0, 3.3.
49. 331.4, 3.3.
50. 341.8, 3.3.
51. 352.2, 3.3.
52. 362.6, 3.3.
53. 373.0, 3.3.
54. 383.4, 3.3.
55. 393.8, 3.3.
56. 404.2, 3.3.
57. 414.6, 3.3.
58. 425.0, 3.3.
59. 435.4, 3.3.
60. 445.8, 3.3.
61. 456.2, 3.3.
62. 466.6, 3.3.
63. 477.0, 3.3.
64. 487.4, 3.3.
65. 497.8, 3.3.
66. 508.2, 3.3.
67. 518.6, 3.3.
68. 529.0, 3.3.
69. 539.4, 3.3.
70. 549.8, 3.3.
71. 550.2, 3.3.
72. 560.6, 3.3.
73. 571.0, 3.3.
74. 581.4, 3.3.
75. 591.8, 3.3.
76. 602.2, 3.3.
77. 612.6, 3.3.
78. 623.0, 3.3.
79. 633.4, 3.3.
80. 643.8, 3.3.
81. 654.2, 3.3.
82. 664.6, 3.3.
83. 675.0, 3.3.
84. 685.4, 3.3.
85. 695.8, 3.3.
86. 706.2, 3.3.
87. 716.6, 3.3.
88. 727.0, 3.3.
89. 737.4, 3.3.
90. 747.8, 3.3.
91. 758.2, 3.3.
92. 768.6, 3.3.
93. 779.0, 3.3.
94. 789.4, 3.3.
95. 799.8, 3.3.
96. 800.2, 3.3.
Table 3
Homology search results for the identification of two specific IgE immunoreactive proteins from the 3rd week culture filtrate of A. fumigatus

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Spot No.</th>
<th>Accession no.</th>
<th>Protein name</th>
<th>Function</th>
<th>% sequence coverage</th>
<th>Experimental Mr(kDa); pi</th>
<th>Theoretical Mr(kDa); pi</th>
<th>Mascot Score</th>
<th>Allergenicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>131</td>
<td>S39831</td>
<td>Hypothetical protein YBL057c (Saccharomyces cerevisiae)</td>
<td>Not known</td>
<td>24.3%</td>
<td>24.1kD; 5.1</td>
<td>23kD; 5.2</td>
<td>55</td>
<td>Not known</td>
</tr>
<tr>
<td>2.</td>
<td>147</td>
<td>Q6BLU6</td>
<td>Unnamed protein product, similar to IPF14568 of Candida albicans (Debaryomyces hansenii)</td>
<td>Not known</td>
<td>28.6%</td>
<td>19kD; 7.7</td>
<td>44kD; pi 4.7</td>
<td>64</td>
<td>Not known</td>
</tr>
</tbody>
</table>

MALDI-TOF analysis of two protein spots from 3rd week culture filtrate led to identification of significant matches (Table 3). Fig. 5a and 5b shows the map of MALDI-TOF spectra obtained for spot no. 131 and 147. MALDI-TOF analysis resulted in putative identification of two allergens as hypothetical protein YBL057c from Saccharomyces cerevisiae and unnamed protein product from Debaryomyces hansenii (similar to IPF14568 of Candida albicans). The identity of these proteins will be established by internal peptide sequences using MS/MS analysis.

At present, proteomics-based studies applied to Aspergillus fumigatus are still in their infancy. In this study, an attempt has been made to screen large number of specific IgE immunoreactive proteins secreted by A. fumigatus (from a clinical isolate of ABPA) by immunoproteomics approach. MALDI-TOF and ESI-MS/MS analysis of these proteins would confirm their identity and facilitate the studies related to structure-function relationship leading to a better understanding of the biology of the fungus. The proteomic database can be of use to study differential protein expression in A. fumigatus under various conditions and during host-pathogen interactions.

ACKNOWLEDGEMENT

We are grateful to Council of Scientific and Industrial Research, Government of India for the financial support (COR0011) and Institute of Genomics and Integrative Biology (IGIB) and The Centre for Genomic Application (TCGA) for MALDI-TOF facility.

REFERENCES

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Identification of novel allergens of *Aspergillus fumigatus* using immunoproteomics approach


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Summary

**Background**

Approximately 20% of the world’s asthmatics are suffering from *Aspergillus fumigatus* (Afu)-induced allergies. The characterization of specific IgE-inducing allergens in allergic aspergillosis patients is fundamental for clinical diagnosis and for immunotherapy.

**Methods**

Immunoproteomics combined with mass spectrometric analysis was used to identify proteins of third-week culture filtrate (3wcf) potentially responsible for Afu-specific IgE immunoreactivity, using pooled sera from Afu-sensitized asthmatics. Their allergenic validity was also tested against patients with allergic bronchopulmonary aspergillosis (ABPA), by two-dimensional (2-D) gel electrophoresis immunoblotting of 3wcf proteins with individual sera from such patients. This helped us to establish a set of candidate allergens, which could be explored further for diagnostic application in allergic aspergillosis asthmatics including ABPA.

**Results**

Peptide mass fingerprint using matrix-assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-TOF-MS) and/or de novo sequencing by MS/MS analysis of the protein spots from 2-D gels led to the identification of a total of 16 allergens of Afu. Eleven of them are being reported as allergens for the first time and five had been reported earlier. Putative isoforms of the proteins Asp f 13 and chitosanase have been observed for the first time. When studied for reactivity of these proteins among patients with ABPA using their individual sera, these patients exhibited sensitization although the pattern was varying. Taken together, these proteins could thus be considered as potential allergens even among patients with ABPA. Three of these proteins viz. the hypothetical protein (# spot no. 5), extracellular arabinase (# spot no. 6) and chitosanase (# spot no. 11) could be major allergens with specific IgE immunoreactivity with six out of eight patients’ sera.

**Conclusions**

The immunoproteomic approach applied to the analysis of culture filtrate proteins resulted in the identification of several candidate allergens, many of them novel, contributing to the catalogue of Afu allergenic proteins, which would facilitate improved serodiagnosis for allergic aspergillosis. In addition, the immunoreactivity of these proteins observed among the patients with ABPA may be potentially useful for its serodiagnosis and opens up further opportunities for the development of personalized immunotherapeutics for patients with ABPA.

**Keywords**

*Aspergillus fumigatus*, allergens, immunoproteomics

Submitted 17 July 2006; revised 16 March 2007; accepted 11 May 2007

Introduction

The mould *Aspergillus fumigatus* (Afu) causes four distinct clinically recognizable forms of hypersensitivity respiratory disorders viz. allergic bronchopulmonary aspergillosis (ABPA), allergic *Aspergillus* sinusitis, IgE-
Efforts of several groups have resulted in identification of allergens from culture filtrate and/or mycelial extracts of \textit{Afu} by conventional purification methods, sequencing of cDNA clones (random screening and immunoscreening) and limited immunoproteomics \cite{1, 2, 3, 6}. Further, only some of the allergens, viz. Asp f 1, Asp f 2, Asp f 3, Asp f 4, Asp f 6, glycoprotein (gp) 55, Asp f 23 and an immunodominant peptide of Asp f 1, have been evaluated for their diagnostic potential \cite{9-11}. In view of the predicted 30,000 proteins of \textit{Afu}, we need to adopt a large-scale approach such as immunoproteomics for identification of a comprehensive catalogue of allergens \cite{12-15}.

Proteomics has a fundamental advantage over the cDNA library approach as it is possible to identify proteins that have been post-translationally modified. In view of the advantages of proteomic approaches over conventional protein purification and characterization, some initial attempts have been made for proteomics of \textit{Aspergillus} \cite{16-20}. Immunoproteomics of mycelial proteins of \textit{Afu} was attempted in a limited way leading to identification and characterization of Asp f 13, a serine protease \cite{21} and enolase \cite{22}.

Earlier, we had reported a comparative two-dimensional gel electrophoresis (2-DE) and immunoblot analysis of \textit{Afu} culture filtrates (first-, second- and third-week culture filtrate) and third-week culture filtrate (3wcf) showed a larger number of specific IgE immunoreactive proteins \cite{23}. Hence, in the present study, 3wcf proteins of \textit{Afu} were used to identify novel allergens using pooled sera of \textit{Afu}-sensitized asthmatics and their allergenic potential was also studied in ABPA patients to establish the candidate allergens, which could be explored further for diagnostic applications in patients with allergic aspergillosis including ABPA.

Materials and methods

Patients and control subjects

The sera were obtained from patients attending Clinical Research Centre, Vallabhai Patel Chest Institute, University of Delhi, Delhi, and healthy controls with written consent using the protocol approved by the institutional human ethics committee of both the institutes.

The subjects (control individual and patients) included in the present study were grouped into three categories; Group A (control or healthy individuals, \( n = 12 \)), Group B (\textit{Afu}-sensitized asthmatics, \( n = 10 \)) and Group C (patients with ABPA, \( n = 8 \)). Sera obtained from Group A, B or C were evaluated for IgE, high eosinophil count, skin positivity, transient pulmonary infiltrates and bronchiectasis and hence their relevance for immunodiagnosis is suggestive \cite{3-6}.

Preparation of culture filtrate proteins

3wcf proteins were extracted from \textit{Afu} (strain 285, isolated from the sputum of a patient with ABPA visiting Vallabhai Patel Chest Institute, Delhi, India) as described previously \cite{23}, for performing \textit{Afu}-specific IgE ELISA, \textit{Afu}-specific IgG ELISA, 2-DE and 2-D-specific IgE immunoblotting.

Two-dimensional polyacrylamide gel electrophoresis

2-DE of the protein from 3wcf was carried out as follows. Isoelectric focusing was performed in 11-cm glass tubes (Bio-Rad, Hercules, CA, USA; inner and outer diameters of 1.5 and 4 mm, respectively). The tubes were filled with 4.5% tube gel containing 8% urea, 2% triton-X 100, 6% ampholine (pH 3.5-9.5) (Amersham, Uppala, Sweden). After a pre-run at 50 and 200 V for 15 min, a total of 600 µg of 3wcf proteins and standard pl marker (Sigma, St Louis, MS, USA) were loaded onto the gel and isoelectric point was determined between Group A vs. B or Group A vs. C or Group B vs. C statistical software packages (http://home.clara.net/sisa/t-test.html). The data were represented as range, mean and SD. When two variables were compared, P-value < 0.05 was considered to be significant.

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Novel allergens of Aspergillus fumigatus 3

Table 1. Clinical and serological details of the patients and control subjects included in the study

<table>
<thead>
<tr>
<th>Patients’ group</th>
<th>Numbers</th>
<th>Age, years (range)</th>
<th>Gender (M/F)</th>
<th>FEV₁ (mean ± SD, range)*</th>
<th>% Eosinophils (mean ± SD, range)</th>
<th>Total IgE (kU/mL) (mean ± SD, range)</th>
<th>Specific IgE (EU/mL) (mean ± SD, range)</th>
<th>Specific IgG (EU/mL) (mean ± SD, range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A (n = 12)</td>
<td>10-62</td>
<td>7/5</td>
<td>3.8 ± 0.4</td>
<td>1.9 ± 0.7</td>
<td>5.4 ± 1.6</td>
<td>1.9 ± 0.7</td>
<td>84 ± 38</td>
<td>27-146</td>
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<tr>
<td>Group B (n = 10)</td>
<td>15-64</td>
<td>6/4</td>
<td>2.86 ± 0.46</td>
<td>7.5-7.8</td>
<td>25-32</td>
<td>2.5-7.8</td>
<td>47 ± 12</td>
<td>190 ± 35</td>
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<tr>
<td>Group C (n = 8)</td>
<td>16-57</td>
<td>7/1</td>
<td>3.25 ± 0.3</td>
<td>1.2-2.9</td>
<td>5.41 ± 1.6</td>
<td>10.5 ± 6.1</td>
<td>5217 ± 1444</td>
<td>5206-1875</td>
</tr>
</tbody>
</table>

P-value
A vs. B < 0.0001; A vs. C < 0.0001; B vs. C < 0.0326
A vs. B < 0.0001; A vs. C < 0.0001; B vs. C < 0.0481
A vs. B < 0.0001; A vs. C < 0.0012; B vs. C < 0.0337
A vs. B < 0.0001; A vs. C < 0.004

*An increase of 200 ml in FEV₁ after inhaling 200 μg salbutamol [2].

Table 2. Diagnostic criteria fulfilled by the eight patients with ABPA

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Patients</th>
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<tr>
<td>Major</td>
<td></td>
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<tr>
<td>Asthma</td>
<td></td>
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<tr>
<td>Presence of transient pulmonary infiltrates</td>
<td>+ + + + + + + +</td>
</tr>
<tr>
<td>ImmediatecutaneousreactivitytoAfu</td>
<td>+ + + + + + + +</td>
</tr>
<tr>
<td>Elevation of total serum IgE level</td>
<td>+ + + + + + + +</td>
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<tr>
<td>Precipitating antibodies against Afu</td>
<td>+ + + + + + + +</td>
</tr>
<tr>
<td>Peripheral blood eosinophilia</td>
<td>+ + + + +</td>
</tr>
<tr>
<td>Central/proximal bronchiectasis</td>
<td>+ + + + + + + +</td>
</tr>
<tr>
<td>Minor</td>
<td></td>
</tr>
<tr>
<td>Expectoration of golden brownish sputum plugs</td>
<td>+ + + + + +</td>
</tr>
<tr>
<td>Positive sputum culture for Aspergillus species</td>
<td>+ + + + + + + +</td>
</tr>
<tr>
<td>Late (Arthus-type) skin reaction to Afu</td>
<td>+ + + + + +</td>
</tr>
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</table>

Patients’ numbers (1-8) correspond to the blot numbers (a-h), respectively in Fig. 4.

Afu, Aspergillus fumigatus; ABPA, allergic bronchopulmonary aspergillosis; FEV₁, forced expiratory volume in 1 s; SD, standard deviation; M, male; F, female; n, number of subjects.

Table 2. Diagnostic criteria fulfilled by the eight patients with ABPA

<table>
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<th>Patients</th>
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Specific immunoglobulin E immunoblot analysis

Proteins (125 kDa) resolved by 2-DE were electrotransferred onto nitrocellulose membranes at 30 V overnight (Schleicher and Schuell, Dassel, Germany). The membranes were blocked with 5% skimmed milk powder in TBST (Tris buffer saline with 0.05% Tween 20 and 0.005% Triton-X-100) at 37°C for 3 h. Test membranes were incubated overnight with pooled sera of 10 Afu-sensitized asthmatics diluted 1:5 in TRIS-+1% BSA+5 mM CaCl₂ at 4°C. Control membranes were similarly incubated with the sera of control patients.

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pooled sera of 10 healthy individuals. All the membranes were further incubated with secondary antibodies (anti-human IgE–chain-specific horseradish peroxidase conjugates from Sigma, 1:1000 dilution in TBST-1% BSA-5 mM CaCl₂ at 37°C and developed with substrate (3,3′-diaminobenzidine). Images were acquired using Gel Documentation System (Bio-Rad).

For Immunoblotting with individual patients’ sera, test membranes were incubated overnight individually with sera of eight patients with ABPA diluted 1:5 in TBST-1% BSA-5 mM CaCl₂ at 4°C. Control membranes were similarly incubated with sera of two healthy individuals.

Selection of specific immunoglobulin E immunoreactive protein spots

Images of 2-DE, ponceau S staining after western blot and immunoblot with pooled sera from A/g-sensitized asthmatics and immunoblots with individual sera from two healthy controls and eight patients with ABPA were compared using Microbial Origin (Version 3) software. Each immunoreactive protein spot was identified in corresponding ponceau S-stained image and 2-D gel. Then, 2-D gel, immunoblot with pooled sera from A/g-sensitized asthmatics and immunoblots with individual sera from two healthy individuals and eight patients with ABPA were compared with a grid after alignment. The SD with respect to molecular mass (MJ) and pl of each specific IgE immunoreactive protein spot in 2-D gel was calculated for the respective protein spot in all the immunoblots. The protein identities were assigned if MJ or pl of any protein spot was found varying within ±5% standard error. Protein identity was not assigned to the spots, which showed ambiguity beyond the above error threshold. The MJ was estimated by comparison with the molecular mass reference marker (Bangalore Genei, Bangalore, India) and pl values were assigned to protein spots by pl the reference marker (Sigma).

Major allergens refer to any antigen that binds to human IgE in > 50% of patients in a clinically sensitive group (http://www.aaai.org/professionals/resources/pdf/allergen_immunotherapy2003.pdf). As the number of patients’ sera screened in the present study was eight, allergens with specific IgE immunoreactivity in > 75% patients have been considered as the major allergens.

Trypsin digestion

Protein spots with specific IgE immunoreactivity with pooled sera of A/g-sensitized asthmatics were subjected to trypsin digestion. Protein spots of interest were excised manually from coomassie-stained gel. Gel slices were trimmed to approximately 1.5 mm³ and destained with 50% acetonitrile (ACN) (400 µL, 15 min each, 3 × ) and soaked in 100% ACN for 5 min to dehydrate the gels. ACN was removed and gel slices were dried in speed vacuum for 20–30 min and were rehydrated with approximately 15 µL of 50% ACN/5% trifluoro acetic acid (TFA) for 25–50 min with gentle agitation. Supernatant was lyophilized at room temperature [24].

Mass spectrometry

Peptides generated by trypsic digestion of each protein spot were reconstituted in 50% ACN with 0.1% TFA for mass fingerprinting. These were spotted on the matrix-assisted laser desorption/ionization (MALDI) plate after mixing with matrix (3,3′-diaminobenzidine) and the peptide mass fingerprint (PMF) was obtained using a Voyager-DE-STR matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometer (PerSeptive Biosystems, Framingham, MA, USA). Spectra were recorded in the reflectance mode using delayed extraction and each measurement was performed using the following parameters: 20 kV accelerating voltage, 72% grid voltage, 175–220 ns delay time and a low laser shots. Peptide mass calibration was applied performed with external mass standards (Calmix 1 and 2; Applied Biosystems). Parallelly, trypsic digests of protein spots were also analysed for MS/MS spectra in a Q-TOF mass spectrometer using a QSTAR Pulsar (ESI-Q-TOF) from PE Sciex (Toronto, Canada) with a nanospray source. Mass spectrometry was obtained at a 1000V spraying voltage. Multiple charged species were subjected to MS MS with collision energy ranging from 30 to 50 eV and MS/MS fragmentation spectra of the selected ions from the trypsic digest of the proteins were obtained to determine peptide sequence de novo.

Protein identification and identification screening

PMFs were obtained by MALDI-TOF MS and used for interrogating NCBI protein database for fungi using Mascot search program (Matrix Sciences, London, UK). The search parameters were partial methionine oxidation, no fixed modification, one missed cleavage and a mass tolerance of 100 ppm. Protein identification (ID) was short listed when the ID was found as first hit with homology to A/g proteins. IDs with homology to fungi other than A/g were not considered in the study. The probability-based
score of 50 was taken as acceptable or as significant score. Protein identification (ID) was short listed for proteins with significant scores and a match from Afu with at least three peptides and/or > 20% sequence coverage. The MALDI-TOF analysis was repeated twice (from two gels) for all the samples and search was performed for each spot.

The proteins that matched with Afu proteins with significant scores after MALDI-TOF-based PMF analysis were assigned the identity. The proteins that matched with fungi other than Afu, in spite of the identification carrying significant score were not short listed. Protein identifications were also confirmed with MS/MS analysis using ESI-Q-TOF (PE Sciex).

The proteins with non-significant scores with MALDI-TOF analysis were also subjected to de novo sequence determination by MS/MS analysis and independently identified. De novo sequence for selected peptide ions of the tryptic digest was derived from MS/MS fragmentation spectra acquired by tandem mass spectrometry and the peptide sequence obtained was subjected to BLASTp search in Afu database (www.ebi.ac.uk/fastas33/proteomes.html) and protein identified.

Results

Clinical and serological details of healthy individuals and patients

Various clinical markers to determine A/u-sensitization were compared between control individuals (Group A), A/u-sensitized asthmatics (Group B) and patients with ABPA (Group C). Significant P-value (< 0.05) was observed when group A vs. B or group A vs. C or group B vs. C were compared for various clinical and serological markers such as FEV₁, % eosinophils, total lgE (Table 1). Significant P-value was also observed when group A vs. B or group A vs. C were compared for Afu-specific lgE or lgG; however, no significant P-value was observed when group B vs. C was compared. Table 2 shows the clinical and serological parameters for eight patients with ABPA included in the study.

Identification of allergens of Aspergillus fumigatus

2-DE and specific IgE immunoblot probed with pooled sera of A/u-sensitized asthmatics and healthy individuals were analysed and 33 specific IgE immunoreactive proteins were subjected to MALDI-TOF-based PMF analysis (Fig. 1). Immunoreactive spots selected for further analysis were detectable by coomassie blue staining of 2-D gels. PMF of the tryptic digests from 33 distinct immunoreactive spots were determined. Seventeen proteins were identified with significant score. Nine of these 17 proteins identified belong to Afu species whereas eight proteins showed homology to proteins from other fungi. MS/MS spectra could be acquired for five of the nine protein spots with match to Afu proteins and identification was further confirmed with the de novo sequence deduced by MS/MS spectra. Out of the remaining 16 protein digests with non-significant identification scores, MS/MS spectra could be acquired for seven of them and database searching showed peptide sequence homology matches with five proteins of Afu with known functions and two hypothetical proteins of Afu. These were grouped with other IDs based on PMF and MS/MS. Thus, out of 16 spots...
Fig. 2. Protein identification and screening strategy. Proteins were identified using peptide mass fingerprints of the tryptic digests. Identifications were confirmed, wherever possible, by MS/MS fragmentation analysis and sequencing. Proteins which were not identified with fingerprints were subjected to predict their possible functional category and thus they may represent novel allergens. Asp f 2, Asp f 13 and catalase, extracellular arabinase, extracellular alkaline protease, chitosanase, UFP0041 domain protein, putative cell wall protein and two hypothetical proteins (Fig. 1a and b, Table 3). BLASTp to all organisms of two hypothetical proteins did not reveal any significant homology to predict their possible functional category and thus they may represent novel allergens. Asp f 2, Asp f 13 and chitosanase are among the intense protein spots observed in the present study. Representative MS and MS/MS spectra of the three novel allergens are shown in Fig. 3.

**Sensitization profile of patients with allergic bronchopulmonary aspergillosis**

Twelve proteins of A. fumigatus were further studied for their allergenic potential in ABPA patients shown in Table 2. The results of these experiments are shown in Fig. 4 and indicated varied sensitization profile. A number of patients with ABPA showing positive sensitivity to the spots corresponding to the sixteen allergens identified have been described in Table 3. Three of the novel allergens, the hypothetical protein (spot no. 5), extracellular arabinase (spot no. 6) and chitosanase (spot no. 11) showed immunoreactivity in six out of eight patients studied and could potentially be novel major allergens (Fig. 4, Table 3). Other identified allergens showing immunoreactivity with < 75% patient sera were classified as minor allergens. The intensity of immunoreactivity of various specific IgE immunoreactive proteins was variable in individual patient sera. Control sera did not show any specific IgE immunoreactivity to 1wc f proteins (data not shown).

**Discussion**

In the present study, we have identified 16 proteins that could be regarded as candidate allergens of *A. fumigatus* with eleven of them being novel. Our study includes identification of proteins such as Asp f 2 and Asp f 13, which were already known as allergens of *A. fumigatus* thus supporting the validity of our experimental approach. Asp f 2, a fibrinogen-binding protein, is a major allergen, with molecular mass 37 kDa, purified from culture filtrate [25] and mycelial extract. Recombinant Asp f 2 has been produced in *Escherichia coli* [26] and *Pichia* [27] and is reported to be 37 and 70-75 kDa, respectively. Banerjee et al. [28], reported that gp 55 may be an isoform of Asp f 2 as it showed complete N-terminal sequence homology to Asp f 2. However, three proteins, spot nos. 2 [55 kDa, 5.3], 3 [55 kDa, 5.4] and 4 [34 kDa, 5.3] were all identified as Asp f 2 in the present study and may represent isoforms or post-translationally modified Asp f 2. Purified Asp f 2 has been shown to bind to specific IgE antibodies in 96% of 25 ABPA patients examined but none in allergic asthma patients [25] whereas in the present study, protein spots identified as Asp f 2 (spot nos. 2, 3 and 4) showed specific IgE immunoreactivity with at least four ABPA patients' sera. Asp f 13, a protease earlier reported as an allergen, was identified from 2-D immunoblot analysis of mycelial extract and SDS-PAGE immunoblot analysis of culture filtrate proteins using pooled patient sera [31]. Thus, our
### Table 3. Protein identification of specific IgE immunoreactive proteins of *Afu*

<table>
<thead>
<tr>
<th>Accession no.</th>
<th>Spot no.</th>
<th>Peptides matched (peptide span)</th>
<th>Sequence coverage (%)</th>
<th>Experimental M&lt;sub&gt;r&lt;/sub&gt;/pI</th>
<th>Mascot score</th>
<th>De novo sequence of peptides</th>
</tr>
</thead>
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<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>AAB48485</td>
<td>1</td>
<td>Catalase</td>
<td>13/13 (75-728)</td>
<td>70.6; 5.30</td>
<td>27</td>
<td>(ND)</td>
</tr>
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<td></td>
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</tr>
<tr>
<td>TAU99330</td>
<td>2</td>
<td>Asp12</td>
<td>8/34 (62-214)</td>
<td>54.2; 5.3</td>
<td>26</td>
<td>(75-728)</td>
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<td></td>
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<tr>
<td>EAL99330</td>
<td>3</td>
<td>Asp12</td>
<td>5/17 (62-214)</td>
<td>23</td>
<td>23</td>
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<td>Asp12</td>
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<td>6</td>
<td>Asp12</td>
<td>3/8 (67-183)</td>
<td>3</td>
<td>3</td>
<td>(74-98)</td>
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<td>2</td>
<td>2</td>
<td>(74-98)</td>
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<td>2</td>
<td>2</td>
<td>(74-98)</td>
</tr>
</tbody>
</table>

Proteins marked with star (*) are already reported allergens of *Afu*.
Proteins marked in bold indicate the major allergens (see text).

(-) indicate no match or low scores to *Afu* proteins.
ND, not determined; *Afu*, Asperillus fumigatus.
Fig. 3. Representative MS (a) and MS/MS (b) spectra of novel allergens (major) of Aspergillus fumigatus identified in the present study namely the hypothetical protein, extracellular arabinase and chitosanase.

observation of allergenicity of Asp f 13 is consistent with the earlier report. We observed weak or no IgE immunoreactivity for two protein spots (presumably isoforms) identified as Asp f 13, with sera of patients with ABPA; however, the diagnostic relevance may be investigated further with more number of patient sera. However, other major allergens of A.f such as Asp f 1, Asp f 3, Asp f 4, and Asp f 9 were not identified presumably on account of experimental limitations.

Allergenicity or immunoreactivity of catalase (involved in degradation of hydrogen peroxide), extracellular alkaline protease (virulence factor with elastolytic activity), UPF0041 domain protein (a protein of uncharacterized protein family), putative cell wall protein, chitosanases (spot nos. 8, 9 and 10) and one hypothetical protein of A.f was observed in ≤60% patient sera and so these were considered as minor allergens.

Among the novel allergens identified in the present study, three viz. the hypothetical protein, extracellular arabinase and chitosanase (spot no. 11) showed immunoreactivity in ≥75% patient sera and were considered as major allergens. The hypothetical protein, spot no. 5 (34.2 kDa, 6.2) showed immunoreactivity with six patients' sera (4a-e, h). Putative extracellular arabinase, spot no. 6 (39.1 kDa, 6.25) involved in degradation of the plant cell wall polysaccharide l-arabinan, showed immunoreactivity with six patients' sera (4a-b, d, f-h) [8, 29, 30]. One of the putative isoforms of chitosanase, spot no. 11 (26.1 kDa, 9.0) showed immunoreactivity with six patients' sera (4b-g) (also see Table 3). The diagnostic
Novel allergens of *Aspergillus fumigatus*

Fig. 4. Specific IgE immunoblots of third-week culture filtrate (3wcf) proteins with individual sera of eight clinically confirmed patients with allergic bronchopulmonary aspergillosis (ABPA). The immunoblots (a–h) correspond to patient numbers (1–8) respectively in Table 2. Patients with ABPA showed varied sensitization profile to thirteen proteins identified and described in Table 3. 3wcf proteins were extracted as in methods and analyzed for specific IgE immunoreactivity with individual sera from eight patients with ABPA. The proteins showing immunoreactivity are marked with arrow and numbered in blots as seen and identified in Fig. 1a. White arrows indicate spots that may represent major allergens. Immunoblot with individual sera from two control healthy individuals did not show any detectable signal with 3wcf proteins (not shown). Ponceau S-stained image of the blot of 3wcf proteins was used to match the two-dimensional electrophoresis (2-DE) gel (Fig. 1a) used for protein identifications. Protein identities were assigned to the immunoreactive spots by comparing the spot coordinates in the respective immunoblot and corresponding Ponceau S blot image.

Potential of these major allergens may be further established with more number of patient and control sera.

The current study using the proteomic approach supported with the genome sequence database for *Af* and integrated with specific IgE immunoreactivity analysis has led to identification of a repertoire of specific IgE immunoreactive proteins from the culture filtrate of *Af*.

This is the first effort to establish identity allergens on a global scale from the culture filtrate of *Af* using immunoproteomic approach. The earlier two studies with...
immunoproteomics of mycelial extracts of \textit{Afu} were focused on identification of salient allergens, viz. Asp f 13 [31] and enolase [22]. Glycosylation and low abundance of proteins affect their efficient proteolysis leaving a large number of tryptic peptides from N-linked oligosaccharides [11]. Sequence information for the genomes of other \textit{Aspergillus} species (completed or under study) will markedly improve the ability to identify more allergens of \textit{Aspergillus} species. The present effort needs to be followed up by a more extensive study to identify the remaining allergens including some of the known major allergens, such as Asp f 1 (18 kDa, 7.2) [7], Asp f 3 (20 kDa, 5-6) [7] and gp55 (55 kDa) [31].

The biological and immunological relevance of iso­forms of Asp f 2, Asp f 13 and chitinase needs to be explored. Similarly, novel allergens identified in the present study may be further explored for their diagnostic and therapeutic potential for allergic aspergillosis patients including ABPA. Varying sensitization pattern of ABPA patients suggest the scope for personalized immunotherapy for allergic aspergillosis patients including ABPA. The role of novel allergens identified in the present study showing sensitivity consistently in Asp f-sensitized asth­matic as well as in patients with ABPA could be explored with respect to pathogenesis in allergic aspergillosis and their potential as antifungal drugs target. Ongoing studies in our laboratory relate to identification of potential allergens of \textit{Afu} with sensitivity specifically in patients with ABPA.

Acknowledgements
This work was carried out at the Institute of Genomics and Integrative Biology, Delhi, India and Centre for Cellular and Molecular Biology, Hyderabad, India. We are grateful to the Council of Scientific and Industrial Research (COR001) and Department of Science and Technology, Government of India for the financial support. Ms Poo­nam Gautam was a recipient of Senior Research Fellow­ship of Council of Scientific and Industrial Research.

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21 Carbrey S, Neville CM, Kavanagh KA, Doyle S. Analysis of major intracellular proteins of \textit{Aspergillus fumigatus} by proteomic methodologies. Biochim Biophys Acta 2006; 1764:1096-104.
29 Campbell S, Neville CM, Kavanagh KA, Doyle S. Analysis of major intracellular proteins of \textit{Aspergillus fumigatus} by proteomic methodologies. Biochim Biophys Acta 2006; 1764:1096-104.
Novel allergens of Aspergillus fumigatus