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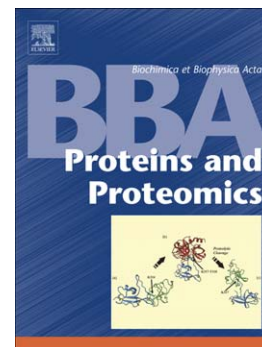
***Curvularia pallescens*, a prevalent aerospore in India**

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Mass spectrometry-based identification of allergens from *Curvularia pallescens*, a prevalent aerospore in India

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Abstract:

The worldwide prevalence of fungal allergy in recent years has augmented mining allergens from yet unexplored ones. *Curvularia pallescens* (CP) being a dominant aerospore in India and a major sensitizer on a wide range of allergic population, pose a serious threat to human health. Therefore, we aimed to identify novel allergens from CP in our present study. A cohort of 22 CP-sensitized patients was selected by positive Skin prick grade. Individual sera exhibited elevated specific IgE level and significant histamine release on a challenge with antigenic extract of CP. First gel-based profiling of CP proteome was done by 1 and 2-dimensional gel. Parallel 1 and 2-dimensional immunoblot were performed applying individual as well as pooled patient sera. Identification of the sero-reactive spots from the 2-dimensional gel was found to be challenging as CP was not previously sequenced. Hence, mass spectrometry-based proteomic workflow consisting of conventional database search was not alone sufficient. Therefore, *de novo* sequencing preceded homology search was implemented for further identification. Altogether 11 allergenic proteins including Brn-1, Vacuolar protease, Fructose-bis-phosphate aldolase were identified with high statistical confidence ($p < 0.05$). This is the first time reporting of any allergens from CP. This kind of proteome-based analysis provided a catalog of CP allergens that would lead an improved way of diagnosis and therapy of CP-related allergy.

Keywords: Allergen, *Curvularia pallescens*, Immunoblot, MALDI TOF/TOF, fungal allergic patients.

1. Introduction:

About 20% of the global population is found to be affected by IgE-mediated hypersensitivity induced by allergen exposure [1]. Both indoor and outdoor fungal spores elicit different respiratory troubles [2]. The World Health Organization and International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Subcommittee (www.allergen.org) has officially recognised 753 allergens till now. The WHO/IUIS allergen nomenclature database (IUIS; www.allergen.org) has been recently updated a list of 109 fungal allergens including 86 Ascomycota allergens and 23 allergens from Basidiomycota originated from 28 fungal species [3,4].

Substantial number of proteins of *Aspergillus* (Asp f 13, Asp f 18, Asp f 34, Asp n 18, Asp fl 18, Asp o 13) and *Penicillium* (Pen c 13, Pen b 13, Pen ch 13, Pen n 13, Pen n 18, Pen o 18) were identified as major allergens [5,6,7,8]. Both major and minor allergens have been accounted from different species of *Candida* [9], *Cladosporium* [10], *Rhizopus* [11], *Alternaria* [12], *Epicoccum* [13].

In the class Ascomycetes, genus *Curvularia* represents one of the reported airborne fungal allergens worldwide, which affects up to 10–16% of patients with nasobronchial allergy, viz allergic rhinitis and asthma [14, 15]. There are few reports from America [16] and Asian countries [17] demonstrating 18–28% prevalence of *Curvularia* sensitization. From different parts of eastern India, *Curvularia* spores were found to be present in the air throughout the year [14,18], and *C. lunata* and *C. pallescens* (CP) were reported as the most ubiquitous species [14,19]. Though allergens like glyceraldehyde 3-phosphate dehydrogenase [20], serine protease [21] and enolase [22] were identified from *C. lunata*, none has been stated so far for CP.

In the present study, we have conducted a two-year aerobiological sampling in Kolkata, a megacity of India to unearth dominant aerospores in the outdoor environment. CP was found

to be one of the prevalent fungal spores, antigenic extract of which showed high allergenic reactivity among the local populace. Till now, to the best of our knowledge, no information is available regarding its allergenicity and its allergens. Hence, we made a first time effort to identify allergens from *CP* through a combinatorial approach consisting of clinical, immunobiochemical and mass spectrometry-based proteomic approach. Our investigation was initiated with Skin Prick Test (SPT) screening of patients having doctor-diagnosed atopic symptoms using *CP* fungal extract, and further confirmation of *CP* sensitivity was performed with *in vitro* immunological tests. Immunoproteomic investigations involved with protein profiling of spore mycelia by SDS-PAGE and 2D-PAGE, followed by corresponding immunoblots to detect IgE-reactive proteins and IgE-binding proteins were identified by mass spectrometry. As the conventional database search precludes identification of peptides not present in the database, *de novo* sequencing combined with homology-driven proteomics became essential to identify proteins from *CP* whose genomes are still not known.

2. Material and Methods:

2.1 Aerobiological and clinical study:

Detailed two-year monitoring (August 2012 to July 2014) of airborne fungal spores has been executed using a Burkard volumetric 7-day sampler (Burkard Manufacturing Co. Ltd., Hertfordshire, UK) in Kolkata, India. Trapped spores were microscopically scanned by a high resolution light microscope (Leitz, Diaplan, Germany) at 400X and counted according to the British Aerobiology Federation guidelines (1995). Several keys, atlases and pictures [23, 24, 25, 26] along with their appearance (color, shape) and morphology (septa) were consulted for spore identification.

Demographic and clinical data of patients who visited a local allergy clinic with seasonal/perennial allergic symptoms were collected retrospectively and analyzed.

2.2 Study subjects:

A cohort of 6 non-atopic individuals and 22 atopic individuals are considered as study subjects.

Inclusion criteria- 18-50 years of age; a history of bronchial asthma, allergic rhinitis, urticaria and eye problems (such as angioedema or conjunctivitis) either alone or in different combination; SPT grade $> +1$ for *CP* extract, total IgE value > 100 International Units, histamine release more than 100 nM and informed consent prior to inclusion, approval from ethical committee of corresponding hospital.

Exclusion criteria- Severe asthma, pregnancy/lactation, malignancy or other severe systemic diseases during skin testing. Corticosteroids and antihistamines were prohibited to avoid masking of severe symptoms. Smokers were excluded.

2.3 Preparation of viable fungal culture and antigenic extract of *CP*:

Airborne fungal spores were trapped by Andersen volumetric air sampler, and identification was verified by Agharkar Research Institute, Pune, India. Then fungi were grown on Potato Dextrose Agar (PDA) plate followed by subculturing in PD Broth (Himedia, India). 1 g lyophilized spore mycelial mat (14 days old) was crushed in liquid nitrogen and soaked in 5 ml 0.1 M Phosphate Buffer (pH 7.2) with constant shaking overnight. After centrifugation, the clear extract obtained was filtered through supernatant through a 0.22 μ m membrane (Millipore) and used for SPT after the estimation of protein concentration by using Bradford reagent (Bio-Rad Laboratories). The extract was also passed through Sephadex G 25 to remove fungal pigments and other non-protein contaminants that may interfere during skin testing.

2.4 Patient selection by SPT and sera collection:

SPTs were performed with 20 μ l of crude antigenic extract (1:10 w/v) placed on the ventral side of the arms and pricked with a sterile lancet [27]. The wheal response was measured after 20 min and graded [28]. Histamine diphosphate (1 mg/ml) and Phosphate buffer (0.01 M, pH 7.2) was used as positive and negative controls respectively.

Blood (5 ml) was collected from each of the 22 *CP*-sensitive patients; serum separated and stored at -20°C . Sera from six non-atopic individuals were also collected to be used as negative control.

2.5 Total IgE and specific IgE estimation:

Total IgE in the serum was estimated using 'PATHOZYME® Total IgE Estimation kit.' Indirect ELISA was utilized to determine specific IgE levels in *CP* susceptible patients according to protocol described [29, 30]. 100 ng/ μ l of crude antigen extract (50 μ l/well) was

used for coating ELISA plates (Nunc), individual patient sera (1:5) used as primary antibody, enzyme-labelled (alkaline phosphatase) monoclonal anti-human IgE (1:1000)-alkaline phosphatase conjugate produced in mouse as secondary antibody and para-Nitro Phenyl Phosphate (pNPP) (Sigma, St Louis, Mo) as liquid substrate. OD was taken at 405 nm in an ELISA reader (Multiscan- Labsystem, Finland). Tests were performed in triplicate.

CP-specific IgE level in each patient was represented as P (OD₄₀₅ of patient)/ N (OD₄₀₅ of normal subject) ratio. In our study P/N ratio ≥ 2.5 was considered as significantly elevated level of IgE.

2.6 Stripped basophil histamine release assay:

Induced histamine release was performed as previously described [31]. Briefly, the peripheral blood was drawn from non-allergic donors and mixed (1: 5 v/v) with 6% dextran in saline containing 0.01 M EDTA and 2% dextrose. After incubation, the leukocyte-rich upper layer was drawn, centrifuged and washed twice with ice cold phosphate buffer saline. The basophils in suspension were stripped off bound IgE by incubation with lactic acid buffer (13.4 mM lactate, 140 mM NaCl, and 5 mM KCl at pH 3.5) for 3 min. Following incubation, cells were washed in HEPES buffer (pH 7.5). Subsequently, the cells were resensitized (with 150 μ l sera with elevated IgE against *CP* allergen plus 4 mM EDTA and 20 mM sodium citrate) and after passive sensitization, those were stimulated with 150 ng of protein in 50 μ l of *CP* extract in HEPES buffer containing 1 mM CaCl₂. After 1-h incubation, the reaction was stopped by the addition of ice-cold 0.9% NaCl (w/v). Following centrifugation, the cell-free supernatant containing histamine was estimated according to the manufacturer's instructions using a kit (Immunotech, France). Histamine release was calculated in nM from the dose response curve with standards. Assays were performed in triplicate.

2.7 Protein extraction, solubilisation, and quantification for proteomic study:

2 g of lyophilized spore-mycelial mat of *CP* was crushed in liquid N₂ and mixed with 1ml of extraction buffer containing 0.5 M Tris-HCl (pH 8), 2% (v/v) Nonidet P-40, 20 mM MgCl₂, 2% (v/v) β-mercaptoethanol, 700 mM sucrose and 1 mM PMSF and was incubated for 1h at 4°C with continuous shaking. After centrifugation supernatant was mixed with equal volume of Tris-HCl (pH 8) buffered phenol. The mixture was homogenized for 30 min at 4°C and finally centrifuged. The proteins in the upper phenol phase were precipitated with five volumes of 0.1 M ammonium acetate in methanol overnight at -20°C and pelleted by centrifugation [32]. After repeated washing, final pellet was dissolved in 100 µl of rehydration (IEF) buffer (without DTT and ampholytes) containing 7 M Urea, 2 M thiourea and 4% CHAPS and stored at -80°C. Protein content was estimated as mentioned previously.

2.8 SDS-PAGE and 2D gel electrophoresis:

Total protein was resolved in 12% SDS-PAGE, and protein bands were observed by (CBB Coomassie Brilliant Blue)-R250 staining. For 2DE protein was processed through Focus Perfect 2D Clean up Kit (G Biosciences, USA) and reconstituted freshly in 125 µl IEF buffer. 1% immobilized pH gradient (IPG) pH 4–7 linear buffer (v/v) (GE Healthcare, Uppsala, Sweden), 25 mM DTT and traces of Bromophenol blue were added. Rehydration loading was done with 7 cm Immobiline Dry Strips (Amersham Biosciences, San Francisco, CA, USA) in a re-swelling tray for overnight. Isoelectric focussing was done using Ettan IPGphor 3 isoelectric focusing system (GE Healthcare) in gradient mode. Next day strips were run in 12% SDS-PAGE following equilibration. Gels were stained with CBB-R250. Both 1D and 2D gels were run in triplicates.

2.9 IgE-specific 1D and 2D immunoblot analysis:

1D-immunoblotting was used to detect the allergenic components in *CP* protein and to determine their apparent molecular weight [33, 34]. Resolved total protein profile was electrotransferred onto a High Bond LFT PVDF membrane (GE Lifesciences, USA) and incubated overnight with individual patient's sera (1:10 dilution in blocking solution). Secondary antibody incubation was done with monoclonal anti-human IgE alkaline phosphatase conjugate produced in mouse (Sigma) in 1:1000 dilutions. Reactive bands were observed by addition of NBT-BCIP (Sigma). For 2D blotting, a similar protocol was followed as 1D western blot. In 2D immunoblot, pooled sera were used along with individual patients' sera as primary antibody. Efficiency of transfer was validated by PonceuS staining. Gels for 2D blotting and CBB staining were run parallel under exactly same protein loads and experimental conditions to assume spots in blot exists in the same coordinates of the stained gel. Further localization of spots was analyzed by comparing stained gel, PonceuS stained blot, immunoblot through ImageMaster™ 2D Platinum 7.0 software (GE Healthcare). 2D immunoblot using pooled sera was also performed thrice to reduce false positive identifications.

Molecular weight of IgE-reactive bands and spots were determined using Quantity One® software (version 4.6.3, Bio-Rad Laboratories, USA).

2.10 Intensity and volume analysis:

Intensity and volume of the IgE-reactive spots were estimated in corresponding 2D gels using ImageMaster™ 2D. The pixel volume of each spot was calculated based on spot intensity and spot area.

2.11 Student's t-test and correlation analysis:

Student's t-test (2-tailed/unpaired) was done to compare the means of total IgE, specific IgE and histamine release between healthy controls and allergic individuals. Correlation and linear regression analysis were performed to study the association among SPT, total IgE, specific IgE, released histamine and number of allergens identified by 2D immunoblot. Statistical analysis was performed using Prism ver. 6 (Graph Pad Prism, San Diego, California, USA).

2.12 Sample preparation for mass spectrometry:

For mass spectrometric analysis protein spots corresponding to 2D, immunoblot were excised from CBB-stained 2D gel and subjected to trypsin digestion as previously described [35] with minor modifications. Briefly spots were destained with 50 mM ammonium bicarbonate and ethanol (1:1) and dehydrated by acetonitrile. Following subsequent reduction and alkylation, hydration and dehydration were done using 50mM ammonium bicarbonate and 100% acetonitrile. In-gel digestion was performed by adding 12.5 ng/ μ l of trypsin (Promega, Madison, USA) and kept overnight at 37°C. After trypsin removal peptides were extracted using 3% TFA and 30% acetonitrile by vigorous vortexing. Further extraction was done by dehydrating the gel piece with 100% acetonitrile. The extracted peptides were vacuum dried completely in a speed vac (Savant, Thermo Scientific, USA) and diluted in 5 μ l of 0.1% TFA in HPLC grade water. The peptides were purified by passing through Ziptip (Thermo Scientific, USA) according to manufacturer's protocol.

2.13 Mass Spectrometric analysis:

The 2.5 μ l samples were mixed with same volume of the matrix solution (0.5mg/ml of α cyano 4 hydroxy cinnamic acid). 2.5 μ l of each sample was applied to MALDI sample plate (Bruker Daltonics, Bremen, Germany) in duplicates. Parent and daughter ion spectra were

acquired by MALDI-TOF/TOF (Autoflex II, Bruker Daltonics, Germany). Mass spectra were recorded in linear mode equipped with a pulsed N₂ laser ($\lambda = 337\text{nm}$, 50 Hz) at 54% power in positive ion mode. High-intensity peaks having good signal to noise ratio (>15) and were manually selected from each MS data are subjected to MS/MS by changing the mass spectrometer to the lift mode.

2.14 Database searching and data interpretation:

Processed peaks were transferred to Biotoools 3.2 (Bruker Daltonics) for inputting data directly into MASCOT search engine version 2.4.1 (<http://www.matrixscience.com>) in the mascot generic format (mgf). Identification of desired proteins from *CP* was performed through MASCOT using the following specifications: proteolytic enzyme - trypsin, maximum of missed cleavage of 1, mass tolerance of precursor ions and fragments- 1.2 Da and 0.5 Da respectively; variable modifications- methionine oxidation and carbamidomethylation of cysteine as fixed modification, peptide charge +1, database limited to NCBI nr (61281535 sequences and 21852449079 residues searched on 07th January 2015) and taxonomy as fungi.

Since the *CP* genome is unsequenced, our identification was based only on MS/MS spectral searches and was considered reliable once a match of at least 2 unique peptides was obtained with 95% confidence ($p < 0.05$) for single protein.

Peptides which did not show homology to any other known proteins in MS/MS-based MASCOT search were subjected to manual *de novo* sequencing. We started at the high mass end of a spectrum that appears to be fragmenting in a well-behaved manner. Low energy collision-induced dissociation (CID) of peptides results in a limited number of sequence-specific fragments. Ions of type y include the C-terminus of the peptide while b-type ions include the N-terminus of the peptide. An intense ion (likely to be a y series ion) is chosen, and sequence was expanded up or down as far as possible in order to reach one or both

termini. The entire process was done using the annotation tool in Flex analysis (version 3.4, Bruker Daltonics) following specifications as described elsewhere [36, 37, 38]. The sequence candidates derived from *de novo* sequencing were pasted in MS-BLAST algorithm (<http://genetics.bwh.harvard.edu/msblast/>) through online submissions. Searches were performed using the settings Programme- blast2p, nonredundant database- nr95 (2014_01); matrix as PAM30MS; “Expect” as 100 and results were determined according to the highest score in the output page.

3. Results and Discussion:

3.1 Aerobiological survey and clinical investigation:

Information on airborne fungi and prevalence of sensitization to fungal allergens is extremely significant for the proper diagnosis, prevention and treatment of allergic diseases. Although recognized as a notable problem, fungal allergy is still poorly defined. A great deal of work has been performed regarding the identification of fungal allergens [5, 39, 40]. In a furtherance of this mode of work, we began with aeromycological sampling, where the aerial spectrum of *Curvularia* (both *pallens* and *lunata*) showed its dominance and consistent presence all round the year in our study zone. Throughout the year, *Curvularia* contributed 1.1 % of total spore load on an average which was one of the dominant contributions. Clinical data compilation depicted that 39% of the investigated patient population had > +1 SPT response to *CP* extract only (Supplementary figure 1) and all of them had high serum IgE titre too. These factors made *CP* a major allergic trigger of the locality, and further detailing of its allergenic aspect became essential.

3.2 Allergenicity assessment of *CP* *in vivo* and *in vitro*:

Demographical and clinical features of the patients enrolled in the study are presented in Table 1.

Study subjects	Age (years)	Sex	Symptoms at diagnosis	SPT grade	Concomitant allergy to other aeroallergens	Concomitant food allergies
1	23	M	AR, BA,U	3	Yes	No
2	25	M	BA,U,EP	3	Yes	No
3	19	M	AR,BA	3	Yes	Yes
4	32	F	AR,BA	4	Yes	Yes
5	23	M	AR,BA,EP	3	Yes	No
6	34	M	AR,BA,U	4	Yes	No
7	35	F	BA,EP,U	3	Yes	No
8	30	F	AR,BA	2	Yes	No
9	21	M	BA,EP	2	Yes	No
10	24	M	AR,U	3	Yes	No

11	21	M	AR,BA	2	Yes	No
12	42	M	AR,BA,U,EP	3	Yes	Yes
13	36	M	AR,BA	3	Yes	No
14	23	F	AR,U,EP	4	Yes	Yes
15	40	M	BA,U	3	Yes	No
16	34	F	BA,EP	2	Yes	No
17	21	M	AR,BA	3	Yes	No
18	22	M	AR,BA,U	4	Yes	Yes
19	34	F	AR,U,EP	3	Yes	No
20	32	F	BA,U	2	Yes	No
21	25	M	BA,U,EP	3	Yes	No
22	23	M	AR,EP	3	Yes	No
23	34	M	-	0	No	No
24	33	M	-	0	No	No
25	26	F	-	0	No	No
26	20	M	-	0	No	No
27	27	F	-	0	No	No
28	24	F	-	0	No	No

Table 1.

The selected 22 patients showed significantly positive (>+1) skin prick test (*in vivo*) with *CP* extract. *In vitro* test such as Histamine release, ELISA and immunoblot were performed with sera from these 22 SPT-positive patients to determine the allergenic potency of *CP* extract. All these patients showed elevated histamine release and specific IgE levels in comparison to controls (figure 1).

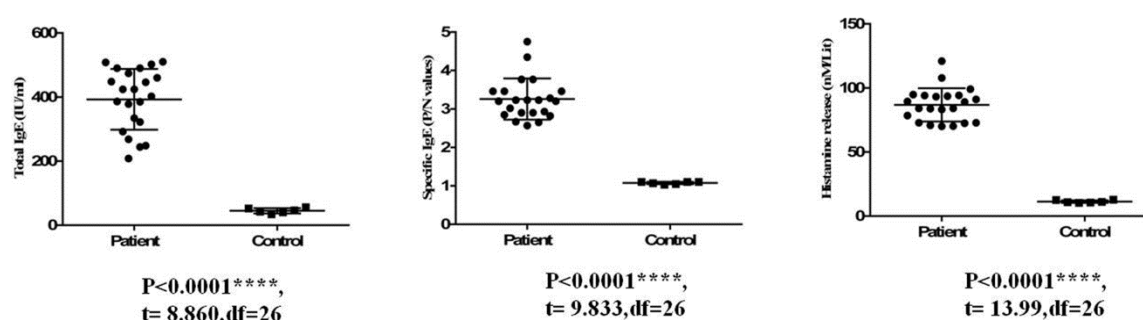


Figure 1.

2-tailed unpaired t-test showed that histamine release upon sensitising basophils with *CP* extract and specific IgE values were upregulated in a statistically significant ($p < 0.0001$) way in *CP*-allergic individuals as compared to controls (figure 1). This authenticated the elevated

atopic response of the patients group to *CP* extract and justified their inclusion in the present study.

The biochemical study includes serological analysis of *CP* proteome resolved in 1D and 2D gel electrophoresis. 1D gel revealed approximately 34 distinct bands in a range from 14.3 to 97.4 kDa (figure 2) which upon western blotting with individual sera demonstrated 7 IgE-reactive bands of approximately 16 kDa, 29 kDa, 35 kDa, 43 kDa, 50 kDa, 65 kDa, 82-95 kDa (figure 2, lane 1-22). On the contrary, control sera did not reveal any bands (supplementary figure 2).

2DE gel resolved the *CP* proteome into almost 205 distinct spots within a range of 14.3-97.4 kDa. 2D immunoblot with pooled sera revealed 15 allergenic spots after matching of 2D gels and corresponding blotted membranes. No spots were observed in the immunoblot using control sera (supplementary figure 2). Spot analysis on 2D gel revealed that all the 15 IgE reactive spots were observed to be consistent in terms of intensity and volume in the replicates (supplementary figure 3). One representative picture of 1D gel (Figure 2A, lane 'T'), 2D gel (Figure 2B) and immunoblot with pooled sera (Figure 2C) is given in figure 2. 1D blot with 22 individual sera is presented in Figure 2A (lane 1-22) and four representative 2D immunoblots using 4 individual sera (patient number 1, 6, 9 and 18) are shown in Figure 2D (from left to right).

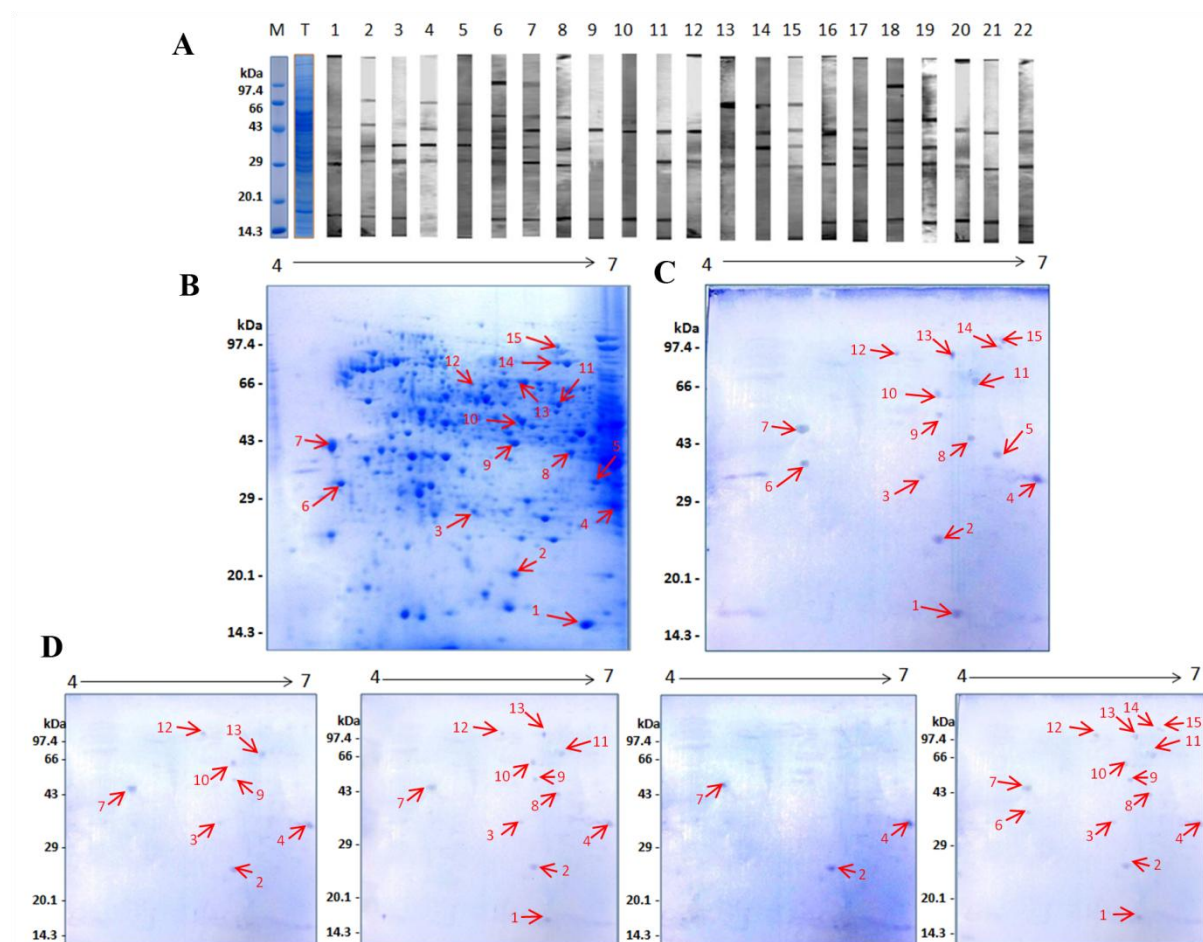


Figure 2.

A single IgE-reactive band exhibited in 1D immunoblot may contain more than one protein or isoforms of a single protein. Hence, for better resolution 2D blot with pooled patient sera was performed. Again, immunoblot using pooled sera does not provide individual reactivity profiles which can vary considerably within patients according to atopicity. To get a better insight, 2D blot with individual patient sera were performed to revealed exact number of allergens reactive in each patient. This was determined by performing cluster analysis that represents the frequency of individual sero-reactive spot as illustrated in figure 3. This binary hierarchical cluster analysis sorted out the entire data into column clusters (representing patient allergenicity profile) and row clusters (representing frequency of *CP* allergens among the sensitised population). In row cluster, spot number 4, 7 and 9 were found in highest

frequency (>80%). Hence, these were considered as immunodominant proteins or major allergens which hold the promise for being used for immunotherapy trials.

When we looked into the individual patient's IgE-binding protein profile in the row cluster, patient number 6 and 18 were detected with 11 and 14 spots respectively. These two patients with +4 SPT grades also had high titre of total and specific IgE as well as a high amount of histamine release. Hence, these two patients were considered as the most sensitive subjects in the study group. Comparatively less number of allergens was found in patient number 1 and 10. Both of their sera exhibited sensitivity to eight sero-reactive spots in the immunoblot. All other 18 patients displayed 3-5 seroreactive spots indicating comparatively low IgE-reactivity.

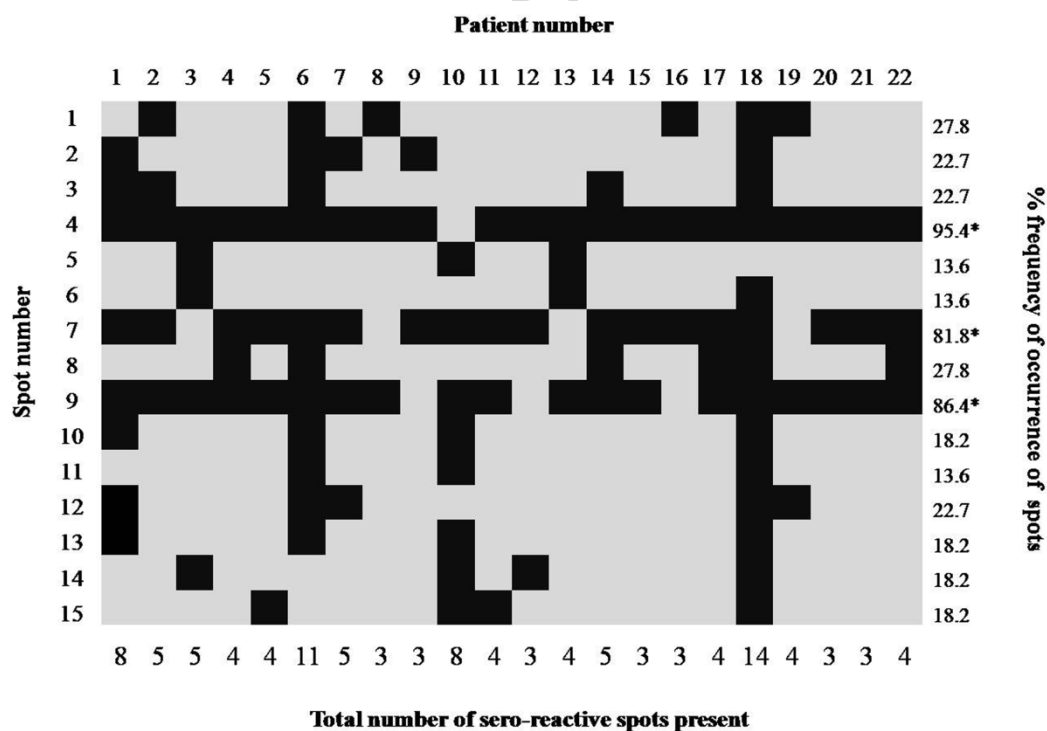


Figure 3.

To find any correspondence between the parameters used for the allergenicity assessment of CP, correlation test was executed (Table 2). SPT grades, specific IgE values, the amount of released histamines and number of allergens found in 2D immunoblot were found to be significantly correlated with each other. This further corroborated the utility of using *in vivo*

(SPT) in combination with *in vitro* tests (ELISA, histamine release, immunoblot) for allergenicity testing of a new source of sensitisation. But correlation was not found between total IgE and most of the above-mentioned parameters. This is probably because total IgE content represents the IgE repertoire generated due to the combined effect of other aeroallergens the patient is exposed to and does not necessarily represent reactivity to *CP* alone.

<i>In vivo</i> and <i>in vitro</i> test parameters	p (two-tailed)	r	R ²	Significance	
1.SPT	1.1.TIgE	0.0547	0.3670	0.5118	NS
	1.2. sIgE	<0.0001****	0.9080	0.9236	S
	1.3. Histamine release	<0.0001****	0.8386	0.8960	S
	1.4. Number of sero-reactive spots	<0.0001****	0.8379	0.5950	S
2. TIgE	2.1. sIgE	0.1793	0.2613	0.4921	NS
	2.2. Histamine release	0.1342	0.2901	0.5840	NS
	2.3. Number of sero-reactive spots	0.0054**	0.5112	0.3136	S
3. sIgE	3.1. Histamine release	<0.0001****	0.8331	0.9029	S
	3.2. Number of sero-reactive spots	<0.0001****	0.8138	0.7279	S
4. Number of sero-reactive spots	4.1. Histamine release	<0.0001****	0.7747	0.6271	S

Table 2.

3.3 Mass spectrometry-based identification of *CP* allergens:

Proteomic platforms have now been extensively used in identifying allergens from a wide number of sources including fungi due its accuracy and efficiency. We have attempted here a similar approach of identifying the IgE reactive proteins from *CP*. *CP* genome being largely unsequenced, a stratified proteomic methodology was used for identifying all of the allergens. Another prevalent *Curvularia* species *C. lunata* has already been explored for its allergens such as glyceraldehyde 3-phosphate dehydrogenase, serine protease and enolase using proteomics approaches. Identification of candidate allergens is a major step towards component resolved diagnosis for designing effective allergen-specific immunotherapy.

The presence of cross-reactivity of allergens amongst related fungal allergens suggests the possibility of using such IgE-reactive elements from a single fungal species to confer resistance through immunotherapy against a wide variety of atopic fungal sources harbouring those allergens. Cross-reactivity studies were performed in seven common *Curvularia* species including *C. lunata* and *C. pallescens*. IgE-specific immunoblotting using *C. lunata* positive patients' sera revealed some common IgE-reactive bands suggesting them to be cross-reactive allergens. Required amount of protein for 50% ELISA inhibition indicated that *C. pallescens* was closer to *C. clavata* and distinct from *C. lunata* regarding shared antigenicity/allergenicity [41].

Characterization of the cross-reactive allergens both at the protein and epitope level would be beneficial in studying cross-reactivity with other fungi. Moreover, identification of cross reactivity among allergens provides new insight in the utilization of current diagnostic tools and therapies for allergy by using a single candidate allergen/hypoallergen against a broad spectrum of allergen sources.

In our study for identification of allergenic proteins, initially, traditional database searching was used to identify highly homologous or identical proteins using daughter ion spectra

generated from MALDI TOF-TOF. As this approach profoundly relies on the presence of annotated genome or protein databases, hence determining correct proteins from unsequenced species like *CP* seems to be a tedious task. The spectra which could not be annotated in this method were manually *de novo* sequenced followed by homology-based search. This complemented the limitations of conventional database searching and added few more peptide identifications thus increasing the sequence coverage and ultimately confidence level of identifications. Identification of unknown proteins typically depends on similarity with homologous proteins from phylogenetically related taxa and is achieved by allowing multiple mismatches through increasing the error window. Here, this method led to identification of almost all the *CP* allergens. The workflow employed here is a simple, time-effective and efficient way for protein identification from unsequenced genomes. Analytical scheme for the identification of proteins is presented in supplementary figure 4.

Most of peptide matches showed homology with proteins from *Pyrenophora tritici repentis*, a fungus which shares an exactly same taxonomic position with *CP* up to Family level. Other matches were obtained with proteins from *Aspergillus*, *Alternaria*, *Penicillium*, *Trichoderma*, *Saccharomyces* and *Leptosphaeria*. All of these genera belong to Ascomycetes group like *Curvularia*. Sequence homology of identified *CP* proteins with these phylogenetically-related fungi has added high degree of authenticity of our identification methodology.

This is also noteworthy that the theoretical mass and isoelectric point of most of the identified proteins matched well with the experimental values. Minor variations can be explained by post-translational modifications (such as phosphorylation, deamidation and so on).

To briefly describe our identification method, the initial step of mass spectrometry analysis was MS/MS based MASCOT search using NCBI nr database. In case of a single spot, if we got at least two unique peptides in MASCOT search to be matched with a particular protein in database, it was considered as validly identified. Spot number 7 and 8 were identified in

this way. For spot number 7 (42.3 kDa and pI 4.96), two unique peptides showed significant match with Vacuolar protease A precursor from *Pyrenophora tritici repentis* with a sequence coverage of 9 %. Spot number 8 (40 kDa and pI 6.5) showed homology with Mannitol-1-phosphate-5-dehydrogenase of *Alternaria alternata* with a sequence coverage of 6 %.

Our second mode of protein identification was the implementation of both MS/MS-based MASCOT search along with *de novo* sequencing followed by MS-BLAST search. We have successfully identified Brn-1 (spot 4, 29.5 kDa and pI 6.85), Formate dehydrogenase (spot 5, 34.3 kDa and pI 6.72), Fructose biphosphate aldolase (spot 9, 42.1 kDa and pI 6.12), ATP synthase subunit beta (spot 10, 49.7 kDa and pI 6.17), Pyruvate decarboxylase (spot 13, 68.2 kDa and pI 6.17), Transketolase (spot 14, 81.9 kDa and pI 5.79) and Peroxidase/catalase (spot 15, 99.2 kDa and pI 6.45) by following this method.

For certain spots such as spot 5, 9, 13 and 15, we observed only one peptide match in MS/MS-based MASCOT search, whereas the ion-score for other peptides were insignificant. Hence, in addition to MASCOT search of one peptide, these spots were identified also by *de novo* sequencing and MS-BLAST. Spot 5, 9, 13 and 15 were identified as Formate dehydrogenase, Fructose-bisphosphate aldolases, Pyruvate decarboxylase and Peroxidase/Catalase respectively with two validly matched unique peptides for each.

For spot 4, 10 and 14, three peptides matches were found, of which two from MASCOT search and the third one from *de novo* sequencing followed by MS-BLAST search. Two peptides of spot 4 showed significant match with the Brn-1 protein of *C. lunata* and *C. tsudae* respectively upon MASCOT search. *De novo* sequencing was done to obtain another peptide sequence, and MS-BLAST search was done to validate this identification (figure 4).

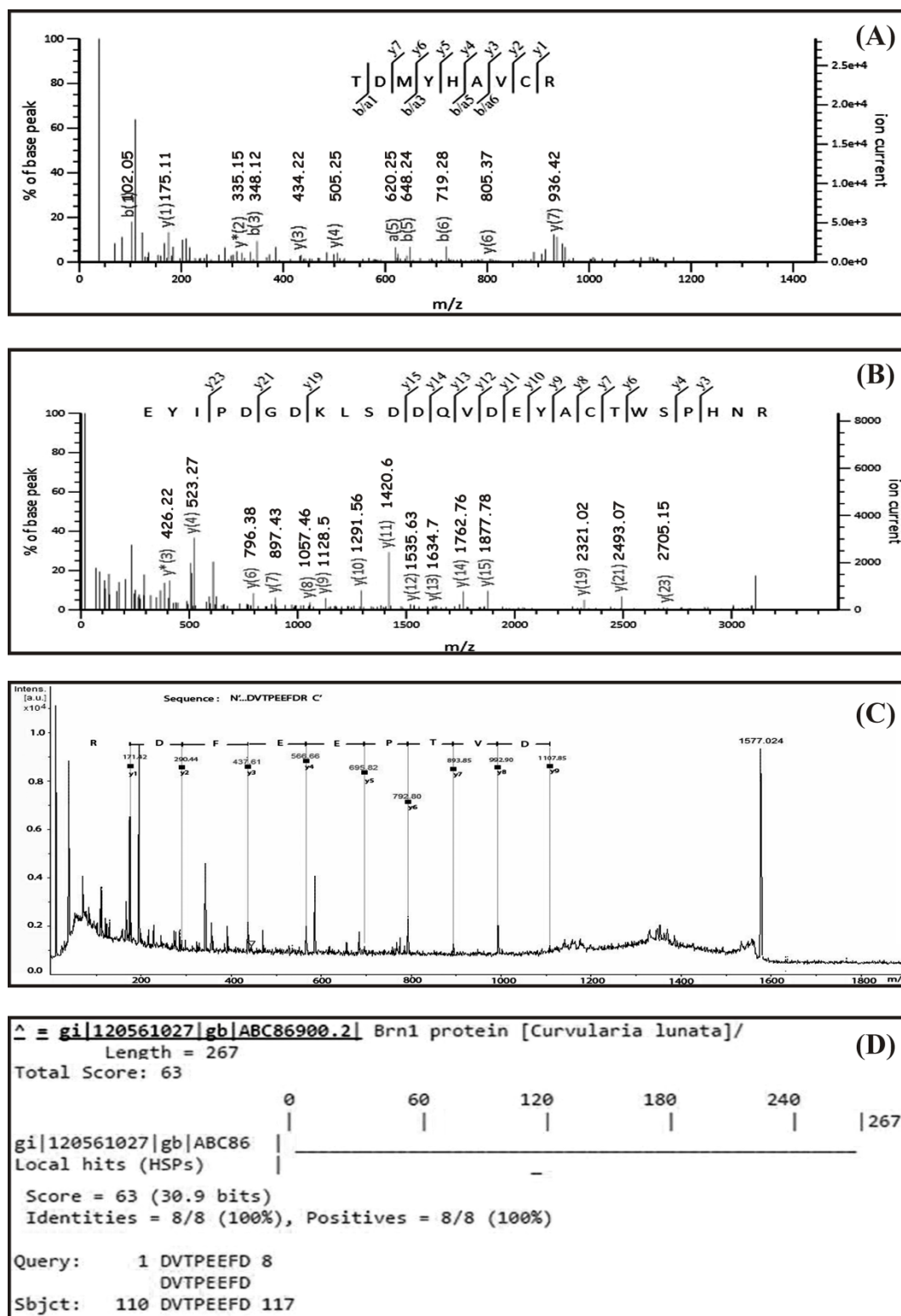


Figure 4.

Two peptides of spot 10 matched with F₁F₀ ATP synthase subunit beta of *Saccharomyces cerevisiae*. Another peptide was *de novo* sequenced and MS-BLAST search revealed reliable

match with the same protein from *Trichoderma atroviridae*. The MS/MS spectra of three peptides of spot number 14 (81.9 kDa, pI 6.4) exhibited significant match with transketolase of *Pyrenophora tritici repentis*.

Spot 6 and 11 did not show any reliable match in MS/MS-based MASCOT search and for that protein identification was solely based on *de novo* sequencing. Two spectra of spot 6 were *de novo* sequenced and finally identified as 14-3-3 protein through MS-BLAST search. Spot 11 (56.6 kDa and pI 6.43) was identified as Phosphoglucanase upon MS-BLAST search of three *de novo* sequenced peptides.

In a nut shell, two proteins were identified by MS/MS-based MASCOT search entirely; two proteins by *de novo* sequencing and another seven proteins were identified by a combination of both. Tandem MS/MS spectra along with their sequences of all the spots are presented in the supplementary figures 5-14.

For spot number 1 (16 kDa, pI 6.62), 2 (21.4 kDa, pI 6.12) and 12 (67.9 kDa, pI 5.77), only one unique peptide showed significant match through MS/MS-based MASCOT search with a hypothetical protein (of *Zymoseptoria tritici*), Asp f 6-like protein (of *Curvularia lunata*) and aldehyde dehydrogenase (of *Coniosporium apollinis*) respectively. Neither we found a second peptide match, nor could perform *de novo* sequencing, so these three spots were not considered as validly identified. Tandem MS/MS spectra along with their sequences of these three spots are presented in the supplementary figure 15. Lastly spot number 3 (29 kDa, pI 5.87) did not show any significant match in MS/MS-based MASCOT search. The fragmented spectra were subjected to *de novo* sequencing but could not be executed properly. As a result, this protein remained unidentified.

We were able to identify 11 out of 15 IgE-reactive proteins from the spore mycelia proteome of *CP*. The protein's identity and other relevant information are listed in Table 3.

All the identified 11 allergens of *CP* were found to have diverse physiological functions required for the survival of the mold. In addition to their usual biological activity, these proteins were found to have special immunological properties that made them potential elicitors of allergic response in atopic individuals. Most of the *CP* allergens identified in the present study have already been reported as major or minor allergens from diverse sources other than fungi. Vacuolar protease A (spot 7) precursors were found to be present in vacuole membrane and involved in vacuolar trafficking and osmoregulation. Vacuolar proteases like serine proteases were already stated allergenic in several fungi such as *Aspergillus* sp. [42, 43], *Cladosporium cladosporioides* [44], *Cladosporium herbarum* [45] and *Curvularia lunata* [21], aspartyl proteases from *Rhizopus oryzae* [46] and so on. Fungal proteases were also known to cause allergic aspergillosis [47]. The role of proteases in eliciting allergic response has also been documented in other indoor allergy such as cat (Fel d 1) and house dust mite (Der p 1, Der p 3, Der p 6 and Der p 9) [48,49]. Protease allergens are particularly important not only for their IgE reactivity but also for enzymatic function. By virtue of their proteolytic activity, the protease allergens were found to create easy access into respiratory system by desquamation of lung epithelia tissue [50]. Mannitol-1-phosphate-5-dehydrogenase (spot 8) has been found to catalyze the interconversion of fructose-6-phosphate and mannitol-1-phosphate in mannitol biosynthesis pathway and was reported as allergenic from *Alternaria alternata* [12] and *Cladosporium herbarum* [10] previously.

Brn-1 (spot 4) is a transcription factor having oxidoreductase activity. As we found this protein as the most prevalent allergen among the *CP* sensitised patients, the successful identification of this spot as gene-regulatory protein Brn-1 has a particular significance. Prior to this, other gene regulatory proteins such as Alt a 7 (22 kDa) and Cla h 5 (22 kDa) have been identified as inhalant allergens from *Alternaria alternata* and *Cladosporium herbarum* respectively [51]. Both of these proteins contain significant homologies to a cytoplasmic

protein of *Saccharomyces cerevisiae* YCP4. Identified peptides of this CP protein, upon Uniprot BLAST search homology showed with YCP4 protein. This protein is involved in negative regulation of transcription and marked as minor allergens showing 7% and 22% positive IgE reactivity respectively. Formate dehydrogenase (spot 5) belongs to D-isomer specific 2-hydroxyacid dehydrogenase family. This protein is an oxidoreductase by function and already reported as allergen from *Aspergillus fumigatus* [52]. ATP synthase (spot 10), which is a mitochondrial inner membrane enzyme involves in ATP synthesis coupled proton transport has not yet been reported as allergen from any mold genus but is a putative allergen from cow dander [53]. Fructose-bis phosphate aldolases were already described as important allergens of *Candida albicans*, Cand a FPA [54]. This 37 kDa major allergen of *C. albicans* possesses 63% homology to *S. cerevisiae* aldolase. Aldolases catalyse the reversible aldol cleavage or condensation and involved in glycolysis, gluconeogenesis and Calvin cycle. Although several bacterial decarboxylases are listed [55] in Allergome database as contact allergens, Pyruvate decarboxylases (spot 13) has been reported for the first time in this study. This is a homotetrameric enzyme that catalyses the decarboxylation of pyruvic acid. Reports are available for allergenic transaldolases in Allfam databases from *Penicillium chrysogenum* [56] but transketolase (spot 14) is marked as allergenic for the first time in our study. Peroxidase/catalases (spot 15) are bifunctional enzymes located in the cytoplasm. Peroxidase from *A. fumigatus* [43], Catalases from *A. fumigatus* [43], *A. versicolor* [57] and *Penicillium citrinum* [58] were reported previously as allergens.

14-3-3 protein (spot 6) belongs to a family of conserved regulatory molecules and can bind a multitude of functionally diverse signalling proteins. Its name refers to the particular elution and migration pattern on chromatography and electrophoresis. Phosphogluconate dehydrogenase (spot 11) is an oxidative carboxylase that catalyses the decarboxylating

reduction of 6-phosphogluconate into ribulose-5-phosphate in the pentose phosphate pathway. Both of these proteins are reported as allergenic for the first time.

Among these 11 identified allergenic proteins, six were already reported allergens from other sources whereas the rest of five, namely Brn-1, 14-3-3 protein, phosphogluconate dehydrogenase, pyruvate decarboxylase and transketolase have been reported as novel *CP* allergens for the first time.

In the present study, we identified three proteins such as Brn-1, Vacuolar protease and fructose bis phosphate aldolase as major allergens of *CP* since these proteins exhibited IgE reactivity in >80% of *CP* sensitized patients. Hence, further investigations are warranted specifically for these three major allergens to develop candidate molecule for clinical diagnostics and attenuated vaccines against *Curvularia* allergy.

Spot no.	Accession no. (NCBI)	Identified Protein	Organism	Unique Peptide	Score	Sequence coverage	Experimental mass/pI	Theoretical mass/pI	Matched peptide Sequence	Functions
MS/MS-based MASCOT search										
7	XP_001941895	Vacuolar protease A precursor	<i>Pyrenophora tritici repentis</i>	2	57/51	9%	42.3/4.96	43.7/5.05	NQDFAEATSEPGLAFAFGR/ YGSGLSGFVSNDVFQIGDLK	Vacuolar sorting and osmoregulation
8	XP_001941485	Mannitol-1-phosphate-5-dehydrogenase	<i>Alternaria alternata</i>	2	37/27	6%	40/6.5	43.6/5.96	VDALLGAVEQAYR/ GIHYVDDLEPYIER	oxidoreductase
*de novo sequencing followed by MS BLAST search										
6	GAA90077*/ CAC20378.1*	14-3-3 protein* N	<i>Aspergillus kawachii</i> *, <i>Trichoderma reesei</i> *	2	79*/61*	6.8%*	34.2/4.75	30/4.89	LDSLSEESYR*/ LAEFASGEK*	cis-acting DNA regulatory element
11	CBF75018.1*/ AAO32398.1*/ AFY04398.1*	Phosphoglucate dehydrogenase N	<i>Aspergillus nidulans</i> *, <i>Saccharomyces bayanus</i> *, <i>Phycus kroberi</i> *	3	74*/67*/61*	8.9%*	56.6/6.43	55/5.86	YFGAHTFR*/ LPANLLQAQR*/ DSFLLELTR*	decarboxylating reduction

MS/MS-based MASCOT search and *de novo sequencing followed by MS BLAST search										
4	ABC 86900.2/ BAA32357/ ABC 86900.2*	Brn-1,partial N	<i>Curvularia lunata/C. tsudae/ C. lunata*</i>	3	23/86/63*	13%, 3%*	29.5/6.85	27.29/7.57	TDMYHAVCR/ EYIPDGDKLSDDQVDEYACTWSP HNR/ DVTPEEFDR*	oxidoreductase
5	XP_001933753/ ERV12119.1*	Formate dehydrogenase	<i>Pyrenophora tritici repentis, Penicillium chrysogenum *</i>	2	35/60*	2%, 2%*	34.3/6.72	40.5/6.21	AILDEYFSGR/ YRPEDLLVHK*	oxidoreductase
9	XP_001938646/ XP_003834549.1 *	Fructose bisphosphate aldolase	<i>Pyrenophora tritici repentis, Leptosphaeria maculans*</i>	2	46/76*	4%, 3%*	42.1/6.12	40.2/5.47	VNLDTDMQWAYLSGIR/ PVVLHTDHCAR*	aldol cleavage/ condensation
10	EDN63437/ EHK47360.1*	F ₁ F ₀ ATP synthase subunit beta	<i>Saccharomyc es cerevisiae/ Trichoderma atroviride*</i>	3	49/79/79*	3%, 2%*	49.7/6.17	54.8/5.52	VVDLLAPYAR/ AHGGFSVFTGVGER/ VAQHLGENVVR*	ATP synthesis coupled proton transport
13	XP_003841651/ AAD16178.1*	Pyruvate decarboxylase N	<i>Leptosphaeria maculans, Aspergillus oryzae*</i>	2	60/111*	1%, 2.5%*	68.2/6.17	63.47/5.76	NPIDIAEYLFR/ CNELNAGYAADGYAR*	decarboxylation
14	XP_001935790	Transketolase N	<i>Pyrenophora tritici repentis</i>	3	51/34/107*	3%, 2.2%*	81.9/6.4	75.3/5.79	YSHEQFGLNR/ AVDFQPPDLGIGEWSGR/ ALPTYKPSDPAIASR*	Metal ion binding

15	EKV06647.1*/ XP_001939675	Peroxidase/ Catalase	<i>Penicillium digitatum*</i> , <i>Pyrenophora tritici repentis</i>	2	67*/33	1.2%*, 1%	99.2/6.45	84.5/6.13	AGVPVPFTPGR*/ HDLVFGHHPELR	Metal ion binding/catalase
MS/MS-based MASCOT search										
1	XP_003854425	Hypothetical protein	<i>Zymoseptoria tritici</i>	1	40	6%	16/6.62	24.9/6.56	QGLPVLDQSGLLSR	-
2	AAQ87929	Asp f 6-like protein	<i>Curvularia lunata</i>	1	75	7%	21.4/6.12	21.3/7.07	HHQTYITNLNALLK	-
12	XP_007776832	Aldehyde dehydrogenase	<i>Coniosporium apollinis</i>	1	35	2%	67.9/5.77	54.25/5.68	VVDTPDFTFNYIR	-
Unidentified (no match)										
3	-	-	-	-	-	-	29/5.87	-	-	-

Table 3.

4. Conclusions:

The present study is a comprehensive report on the allergenic importance of *CP*, a fungus that causes severe respiratory sensitisation among the atopic population of India. This study is a combinatorial approach that includes preliminary clinical and cross-sectional study to screen for the presence of IgE antibody against *CP* allergens among a large number of allergic patients, which was followed by proteomic investigation to identify these allergenic proteins using state of the art mass spectrometry technique. This is a preliminary report that identifies new *CP* allergens for the first time. This information will open up further avenues to purify and characterise individual allergenic components from this mold to improve component resolved diagnosis and immunotherapy of fungal allergy.

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Figure legends:

Figure1. Estimation of total IgE, specific IgE and basophil histamine release by 22 patients and six controls. Values of patient and control group are compared through 2-tailed unpaired t-test. Values are represented as mean of triplicates.

Figure2. SDS-PAGE and 2D PAGE of total protein of CP and corresponding immunoblots.

(A). Molecular weight marker (M), Total protein of CP (T) in 12% SDS-PAGE and 1D blot with 22 individual sera (lane 1-22) (from left to right)

(B). 2D gel of spore-mycelial proteome of CP,

(C). 2D immunoblot using pooled sera of patients.

(D). Four representative 2D immunoblots using 4 individual sera (patient number 1, 6, 9 and 18; from left to right).

Figure3. Number and frequency of IgE-reactive spots in 2D immunoblot shown by a heat map. Heat map constructed in MS-EXCEL, 2010. Major allergens are marked (*) at the extreme right column.

Figure4. MS/MS spectra for spot number 4. (A) and (B) MS-MS spectra of m/z 1152.796 and 3111.037 respectively, (C) *de novo* sequencing of m/z 1577.024 and (D) snapshot of MS-BLAST search.

Table captions:

Table1. Demographical and clinical data profile of 22 patients and six control subjects.

AR=Allergic Rhinitis, BA=Bronchial Asthma, U=Urticaria, EP=Eye Problem (Angioedema/Conjunctivitis).

Table2. Correlation analysis between *in vivo* (SPT) and *in vitro* (histamine release, specific IgE. Total IgE and immunoblot) test values. p= significance (two-tailed), r= correlation co-efficient, R²= regression co-efficient. S= Significant, NS= Not Significant.

Table3. The result of mass spectrometric analysis and database search. Results derived from *de novo* sequencing are marked (*). Newly identified allergens are marked (⌘).

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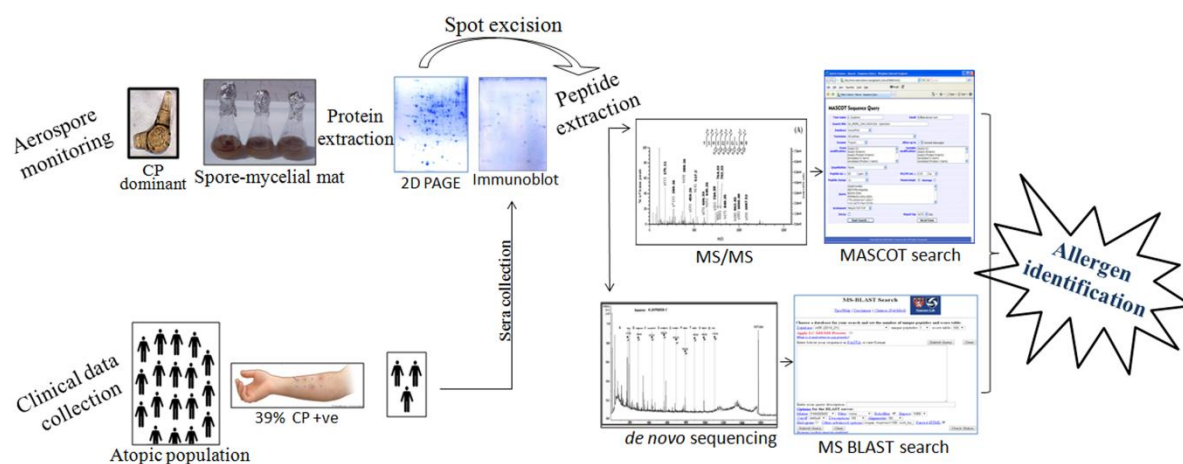
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Graphical abstract



Highlights

- *CP* was found to be a major allergenic trigger in the population of Kolkata, India.
- *In vivo* and *in vitro* allergenicity tests confirmed its allergenic potency.
- 11 potent allergens were identified from *CP* by using proteomic tools.
- This study presents allergenic proteins from *CP* for the first time.