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ORIGINAL ARTICLE

SERO-PREVALENCE OF MAJOR FUNGAL ALLERGEN 'ASP F 3' IN INDIAN POPULATION: POSSIBILITY FOR AN IMMUNODIAGONOSTIC MARKER ANTIGEN

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Abstract*: The prevalence of allergic disorders due to fungal exposure has been rising significantly, throughout the globe. However, when compared to pollen or food allergens, there is a scarcity of molecular studies focusing on fungal allergens. Aspergillus fumigatus is a common airborne fungus that can cause serious infections in immunocompromised individuals in terms of respiratory allergy, asthma, invasive aspergillosis (IA) and allergic bronchopulmonary aspergillosis (ABPA), etc. Environmental surveys indicate that ubiquitous spores of A. fumigatus are getting constantly inhaled by people all around. As a consequence, disease occurs predominantly in the lungs, although dissemination to virtually any organ may occur in the severely predisposed people. The Asp f 3, is an immunoglobulin E (IgE) reactive allergenic protein, having an important role in the pathogenesis of ABPA and IA, both of which affect the respiratory system. The present study aims to understand the sero-prevalence of major fungal allergen Asp f 3 in Indian population.*

For this, the Asp f 3 cloned 'pBBL-TEV' vector was transformed into chemically competent E. coli cells, leading to overexpression. The recombinant protein was isolated and purified by Ni-NTA chromatography. The extracted Asp f 3 protein sample was run through Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) and its IgE reactivity was studied by immunoblotting. The IgE- binding strength of Asp f 3 is comparable to that of Asp f 1, which has already been established as a major allergen, suggesting that rAsp f 3 as a major fungal allergen in the context of Indian population.

Keywords: Fungal allergen, *Aspergillus fumigatus*, recombinant Asp f 1, IgE-reactivity, sero-prevalence, Indian population

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1. INTRODUCTION

Allergy, specifically Type 1 hypersensitivity, occurs when the immune system reacts to an 'allergen', resulting in various allergic symptoms. While most people remain unaffected by these allergens, individuals with atopy (a genetic predisposition) face serious risks, including potentially life-threatening reactions. An allergen can be defined as a non-pathogenic antigen which is capable of triggering a potent immunogenic response. These might be fungal allergen, food allergen, pollen allergen, medications and many more. The hallmark of allergic reactions is an abnormal increase in serum immunoglobulin E (IgE) levels after exposure to allergens.

Many known species of pathogenic fungi are potent sources of allergens [1]. Fungi constitute a major part of the bioaerosol. Approximately 20-30% of atopic population in developed countries is allergic to fungi [2]. Allergenic fungi mostly belong to genera *Aspergillus*, *Alternaria*, *Cladosporium* and *Penicillium* [3, 4].

Fungal allergy usually appears as IgE-mediated type-1 hypersensitivity and manifestations may include asthma, rhinitis, atopic dermatitis or allergic conjunctivitis but in some cases, it may generate type 2 or type 3 hypersensitivity as well. *Aspergillus fumigatus* is the main causative agent of respiratory fungal allergy. *Aspergillus fumigatus* is a filamentous fungus found in the environment. It produces a variety of antigens that can trigger immune responses in humans. Among these, Asp f 3 is a cell wall component [5] that has been implicated in both allergic and invasive aspergillosis. While invasive aspergillosis is a life-threatening condition, allergic aspergillosis can also cause significant morbidity. The Asp f 3 protein plays a crucial role in the pathogenesis of allergic bronchopulmonary aspergillosis (ABPA) and invasive aspergillosis (IA), both of which affect the respiratory system [6]. ABPA is an allergic response to '*A. fumigatus'* antigens, while IA is a severe infection, primarily seen in immunocompromised individuals. Given its relevance in fungal infections, Asp f 3 has garnered interest as a potential immunodiagnostic marker. Detecting specific antibodies against Asp f 3 can aid in diagnosing ABPA and IA. Serological tests targeting Asp f 3 antibodies may provide a non-invasive and efficient way to identify fungal infections, especially in high-risk populations [6].

In recent years, Asp f 3 has emerged as a promising candidate [7]. Studies from other regions have reported its presence in both healthy individuals and those with clinical manifestations of aspergillosis [8]. However, data specific to the Indian population are scarce. Understanding the sero-prevalence of Asp f 3 in India is essential for tailoring diagnostic strategies and optimizing patient management [9].

The accurate diagnosis of *A. fumigatus* infections is crucial for timely intervention and improved patient outcomes. Traditional diagnostic methods, such as culture and microscopy, have limitations in terms of sensitivity and specificity. Therefore, there is growing interest in identifying reliable immunodiagnostic markers.

Aspergillus fumigatus is a common airborne fungus that can cause serious immune reactions and infections in immunocompromised individuals. Environmental surveys indicate that ubiquitous spores of *A. fumigatus* are getting constantly inhaled by people all around. As a consequence, disease occurs predominantly in the lungs, although dissemination to virtually any organ may occur in the severely predisposed people. Inhaled spores in normal healthy subjects are generally eliminated by the innate immune cells. Thus, inhalation of conidia by immune-competent individuals rarely has any adverse effect and remains undetected. Thus, until recent years, *A. fumigatus* was observed as a weak pathogen responsible for allergic forms of the disease.

However, in the recent decades, increase in the use of immunosuppressant to treat various illness has increased

the number of immunosuppressed patients leading to drastic rise in the prevalence of more complex forms of aspergillosis (viz., allergic aspergillosis, that usually affects people with asthma, cystic fibrosis and chronic forms of aspergillosis causing chronic obstructive pulmonary disease (COPD), sarcoidosis and tuberculosis [2, 3, 10]. The Asp f 3 protein, derived from *Aspergillus fumigatus* has been studied for its potential as an immunodiagnostic marker.

The recombinant Asp f 3 protein has been investigated as a potential diagnostic marker for aspergillosis in humans. Aspergillosis is a fungal infection that can be particularly problematic for patients undergoing hematopoietic cell transplantation [11]. During infection stage, the fungal redox homeostasis can be challenged by reactive oxygen species (ROS). Asp f 3, a peroxiredoxin, was found to be essential for causing invasive aspergillosis (IA) in mice. In vivo, Asp f 3 acts as a sensor for ROS generation. It plays a role in activating redox-responsive genes, including those involved in gliotoxin biosynthesis and the ROS defense machinery. Asp f 3 serves as an intracellular redox sensor for target proteins [12]. Asp f3 compensates for the loss of iron-dependent antioxidant enzymes. It plays a crucial role in oxidative stress resistance and virulence. Additionally, a functionally redundant Asp f3-like protein was identified [13]. But the present treatment of *Aspergillus* allergy is mainly based on corticosteroids, NSAIDs and antihistamines which can alleviate the symptoms but are transient and may pose several health threats upon long term use. Hence, allergen specific immunotherapy (SIT) can be employed as a targeted and long-lasting approach. SIT reverses the sensitization by introducing gradually increasing doses of allergen to which the subject is allergic. It diverts the immune response from producing allergy inducing IgE antibodies towards blocking IgG antibodies [9].

Fungal allergens are now getting characterized for various downstream applications which culminate in designing safe and effective next generation vaccine. International Union of Immunological Society (IUIS) has registered twenty-six *A. fumigatus* allergens till date. Asp f 3 is one of the important allergens from *A. fumigatus*, showing high sero-reactivity among populations with fungal allergy. Obtaining the epitope information of this important allergen will be helpful for both diagnostic and therapeutic approaches. By modifying and reconstituting the IgE binding regions of Asp f 3 we can design safe and effective hypoallergenic vaccine. Thus, specific immunotherapy (SIT) can be a promising direction of therapeutic intervention for *Aspergillus* mediated allergy [14].

Asp f 3 has gained attention as a potential marker. Research from various regions indicates its presence in both healthy individuals and those affected by aspergillosis. However, data specific to the Indian population remain limited. Investigating Asp f 3's sero-prevalence in India is crucial for customizing diagnostic approaches and improving patient care.

The objective of the present study is to assess the sero-prevalence of major fungal allergen Asp f 3 in Indian population.

2. MATERIALS AND METHODS

The full-length cDNA of Asp f 3 are already cloned in 'pBBL-TEV' vector. Cloning and recombinant expression already done in the lab. IgE reactivity data of the allergens Hel a 6 (allergen from *helianthus annuus* or sunflower pollen) and Asp f 1 (from *Aspergillus fumigatus* fungi) was obtained from GS Lab. Purified allergen nHel a 6 (from *Helianthus annuus*) was gifted by Prof. Amiya Kumar Panda, Vidyasagar University.

Patient sample collection

Patients who are affected by respiratory allergen screened with fungal sensitization and the residual sera of these patients were collected under the supervision of medical professional, B. R Singh Hospital, Sealdah and Drs. Tribedi & Roy diagnostic laboratory, Park Street, Kolkata. For controls, sera from non-allergic healthy individuals and respiratory sera samples from allergic individuals with other than fungal sensitization was also collected. The entire study was approved by the human ethics committee of the hospital as well as Presidency University. Written consents were obtained from the human subjects participated in the study (Ref.no. PU-HEC-S/GS/HIS/2024/001 dated 1st April,2024 and the approval Ref. no. PU-HEC-S/GS/HIS/2024/001/003 dated 23rd April, 2024).

Preparation of competent cells:

Table 1A: Composition of TFB1 buffer (pH 5.8)

Components	Amount per litre	Components	Amount per litre
100 mM RbCl	12.1 g	10mM $CaCl_2$	1.1 g
50 mM $MnCl_2.4H_2O$	9.9 g	Glycerol	15 ml
30 mM potassium acetate	2.9 g	Distilled water	959 ml

After mixing the components of buffer, it was sterilized by filtration. The pH was adjusted to 5.8 before adding the entire distilled water.

Table 1B: Composition of TFB2 buffer (pH 6.8)

Components	Amount in litre
10 mM MOPS	2.1 g
10 mM RbCl	1.2 g
75 mM $CaCl_2$	8.3 g
Glycerol	15 ml
Distilled water	973.4 ml

The TFB1 and TFB2 buffer solutions were prepared for competent cells preparation as per composition depicted in table 1A and 1B.The *E.coli* (*Rosetta* strain) bacterial cells, cloned with recombinant rAsp f 3 gene were streaked on Luria Agar plates and incubated overnight at 37˚C. A single colony was taken and inoculated in 20 ml Luria broth along with antibiotics-ampicillin and chloramphenicol (each 100 mg/ml concentration) and incubated overnight at 37˚C in an incubator under shaking conditions. The growth culture was again used to inoculate100 ml of freshly prepared Luria broth and was allowed to grow in incubator-shaker until optical density (OD) at 600 nm reached 0.5 (for nearly 3 hours). Then the culture was cooled on ice for 5 minutes and was transferred to centrifuge tubes.

The media was centrifuged for 5 minutes at 4000 g (4˚C) and the supernatant was carefully discarded, keeping the cells on ice. The cell pellet was gently resuspended in 30 ml of cold TFB1 buffer (for 100 ml culture) and

was kept on ice for nearly 1.5 hours.

The cell suspension was again centrifuged for 5 minutes at 4000 g (4˚C) and the supernatant was again discarded carefully; keeping them on ice. Again, the cells were resuspended in 4 ml ice-cold TFB2 buffer and aliquots were made of it on sterile micro centrifuge tubes. The aliquots were stored at -20◦C until further use.

Transformation of *E. coli* Rosetta competent cells with allergen clone:

The following steps were conducted for transformation:

At first, 40 µl competent *E. coli Rosetta* were taken from -80˚C, and 9 µl sterile distilled water (dH_2O) and 1 µl Asp f 3 were taken and added to Rosetta cells. Then vials were tapped for 2-3 seconds and kept on ice for 30-40 minutes. Water bath was set at 42˚C and the vial was heat shocked for1 minute and immediately put it on ice.

After that 1ml LB broth (Luria–Bertani medium) was added and kept it at 37˚c for 1 hour in shaking condition. The vial was centrifuged for 10 minutes at 150 rpm. Most of the LB (around 800µl) was discarded and the rest was used to resuspend the cell pellet in media. Then 80 µl of it was spread on LB-Amp-Chl plate and incubated at 37˚covernight.

Preparation of 20 ml LB-Amp-Chl plate

It was made by mixing 20 ml LB broth, 20 µl Ampicillin and 20 µl Chloramphenicol.

Seed culture of transformed *E. coli Rosetta* cells

A colony from the LB-Amp-Chl plate was picked and cultured in a 20 ml LB broth. The culture tube was incubated overnight under 37°C in a shaker.

Isopropyl –β-D-1-thiogalactopyranoside (IPTG) induction

1 ml seed culture was inoculated into 100 ml LB broth and 10 µl of ampicillin and 10 µl of chloramphenicol was added to it. It was incubated in incubator-shaker at 37˚C for 4 hours. Then 250 µl 0.5 mM and 0.25 mM IPTG was added to it and incubated at 20˚C for overnight in shaker-incubator. It was then stored at 4˚C.

Recombinant protein isolation and purification by Ni-NTA chromatography

i. The following steps were conducted: for isolation and purification of recombinant protein:
ii. The IPTG induced cells were divided into two centrifuge tubes and centrifuged at 5000 rpm for 20 minutes.
iii. The pellet was collected and stored at -20˚C until further use.
iv. Meanwhile, the buffers essential for purification was prepared. The composition of the buffers are stated in the table 2 and table 3.
v. All the buffers prepared were stored at 4˚C until further use.
vi. 10 ml of prepared lysis buffer was taken in two centrifuge tubes containing the cell pellet and was vortexed. It was then kept on ice for 1hour.
vii. The centrifuge tube was then kept on ice and the cells were lysed in a sonicator at 80% power rate for 30 minutes. (The pulse was kept on for 30 seconds and was again off for 60 seconds).
viii. The lysed cells were then centrifuged for 12 minutes at 6000 rpm and the supernatant was collected without disturbing the cell pellet and was kept on ice.
ix. The cell lysate was added to the Ni-NTA column and incubated overnight in cold-room.
x. Next day, the column was taken and the cap was opened to elute the unbound proteins out and was

collected in a centrifuge tube and was marked as 'Flow through'.

xi. Then wash buffer was added to the column keeping the cap closed and then the cap was opened and was collected in a centrifuge tube and was marked as 'Wash 1'. The column was again washed and the flow trough was again collected in centrifuge tube and marked as 'Wash 2'.

xii. Then Elution buffer 1(200 mM Imidazole) was added to the column and was incubated for 30 minutes at 4◦C. after that, the elution was collected and was marked as 'Elution'.

xiii. The protein concentration in the eluted fraction was then measured using Nanodrop Spectrophotometer (Thermo Fischer, Scientific, USA).

xiv. The oligomeric status of recombinant allergen rAsp f 3 was determined using reducing vs non-reducing condition in 15% SDS PAGE.

Table 2: Composition of Lysis buffer (30 ml)

Component	Amount (ml)
25 mM Tris (stock 100 mM)	7.5
300 mM NaCl (stock 3 M)	3
10 mM Imidazole (stock 1 M)	0.3
5% Glycerol	1.5
Distilled water	17.7

Table 3: Composition of Elution buffer (150 mM Imidazole, 30 ml)

Component	Amount (ml)
25 mM Tris	7.5
300 mM NaCl	3
200 mM Imidazole	7.5
5% Glycerol	1.5
Distilled water	10.5

Gravity column Preparation

30 ml of 20% ethanol was passed through Ni-TA column then 30ml of distilled water was also passed through it. 1 ml of Agarose-Ni beads were added to the column and the cap was let open to pass the ethanol. 10 ml lysis buffer was added to it and allowed to pass through. When a small amount of it was left, the cap was closed and it was kept at 40˚C for 30 minutes. Then the cap was removed to discard the lysis buffer.

IgE (Immunoglobulin E) specific immunoblot

The extracted Asp f 3 protein sample was run through Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) and transferred on to PVDF membrane, using semi dry transblotter (ATTO, Japan). Membrane transfer of the protein from the gels was confirmed by staining the membranes with Ponceau S (Sigma-Aldrich, USA) for 5 minutes.

Immunoblot assay

The membrane was cut as per the five lanes of the gel and were placed in 8-chamber plate. The buffers essential

for the assay was prepared. The strips were washed with gold buffer for 10 minutes to remove Ponceau S stain. 1.5 ml blocking buffer was added to each strip and added incubated overnight in cold room under gentle rocking in a rocker. Then blocking solution was discarded and washed with gold buffer for 5 minutes. Then primary antibody human sera (1:10 dilution v/v) was added and incubated overnight in cold room with gentle rocking. The strips were then washed 3 times with gold buffer for 15 minutes each time. Secondary antibody (1:1000 v/v) was added to each strip and kept for 2 hours. The strips were again washed 3 times for 10 minutes each under gentle rocking. Then substrate NBT-BCIP was added to each and band development was observed.

3. RESULTS

Transformation of E. coli Rosetta cells and Recombinant expression of recombinant allergen rAsp f 3

After overnight incubation, colonies appeared on the Luria agar plates containing ampicillin and chloramphenicol. From the observation it can be interpretated that the *E. coli* cells were successfully transformed after competence generation. Distinct single colonies were visible and one such colony was used as an inoculation for the seed culture.

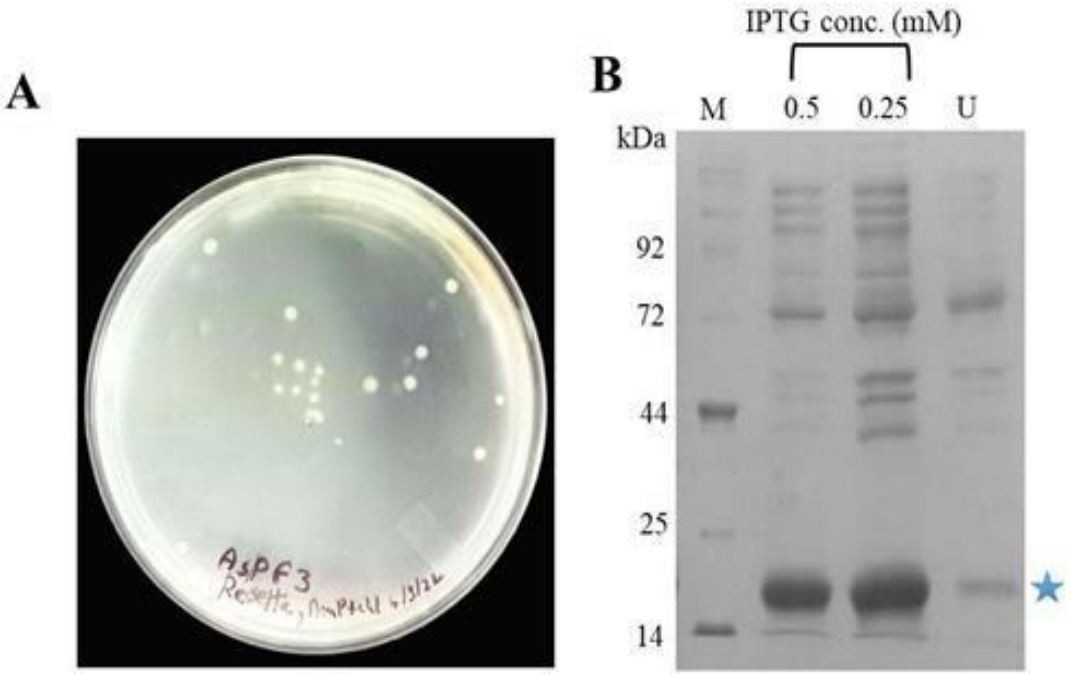

Figure 1. Recombinant expression of rAsp f 3: (A) LB-agar plates containing Ampicillin 100 µg/ml showing transformed colonies of *E. coli* Rosetta cells containing pBBL vector with full length Asp f 3 insert. (B) 12% SDS-PAGE showing induction of recombinant Asp f 3 (rAsp f 3) after addition of 0.5 mM and 0.25 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG). Uninduced (lane U) cell extract was run as control.

The total protein from the Isopropyl β-D-1-thiogalactopyranoside (IPTG) induced and uninduced cultures of rAsp f 3 transformed Rosetta was run on a SDS polyacrylamide gel (12% resolving). Figure 1 shows that rAsp f 3 was present in much greater amounts in the IPTG-induced cells (lane 0.5 and 0.25) compared to the uninduced ones (lane U). The expression level was shown higher in 0.25 mM. Hence, we adhered to using that concentration.

Purification of the recombinant allergen rAsp f 3

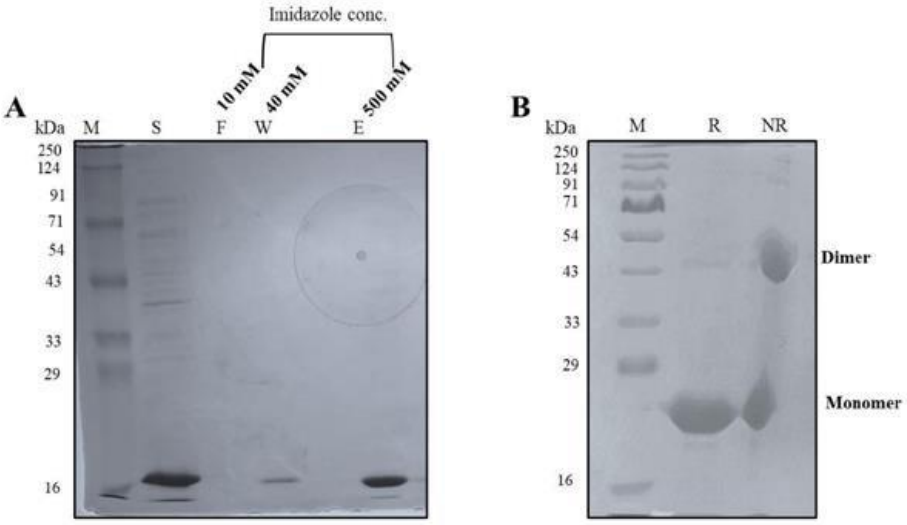

Figure 2. Purification of rAsp f 3. (A) 12% SDS-PAGE showing purified extract of rAsp f 3 protein obtained in elution (E). The molecular weight of the protein was nearly 19kda compared with molecular marker. (B) 15% SDS-PAGE showing oligomeric status of rAsp f 3 appeared as dimer (44 kDa) when run under non-reducing condition without β-mercaptoethanol.

rAsp f 3 displayed IgE reactivity

Western blot was performed using serum from patients allergenic towards Aspergillus fumigatus as the source of IgE. The secondary antibody was monoclonal human anti-IgE conjugated with alkaline phosphatase.

Figure 3 shows the membrane strips after Western blot. It can be inferred that the reaginic antibody, IgE responsible for allergenic hypersensitivities can bind to the rAsp f 3 protein. This highlights the immunoreactivity of rAsp f 3 and also indicates it to be a potent allergen in reality. The blot was performed using serum from a healthy individual yielded no band (Figure 3, lane H). However, bands (corresponding to 19 kDa) could be visualized in the blots performed with patient sera (Figure 3, lanes P1-P8).

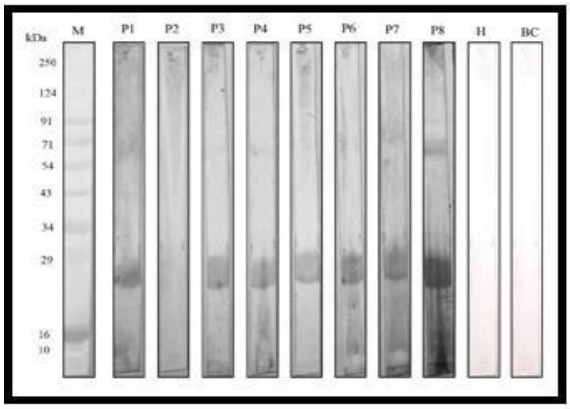

Figure 3. IgE specific Western blot set-1. M: MW ladder (Puregene, Genetix, New Delhi). P1-P8: IgE specific Western blot using sera from patients allergenic to Aspergillus fumigatus. H: Control, healthy serum, BC: Buffer control.

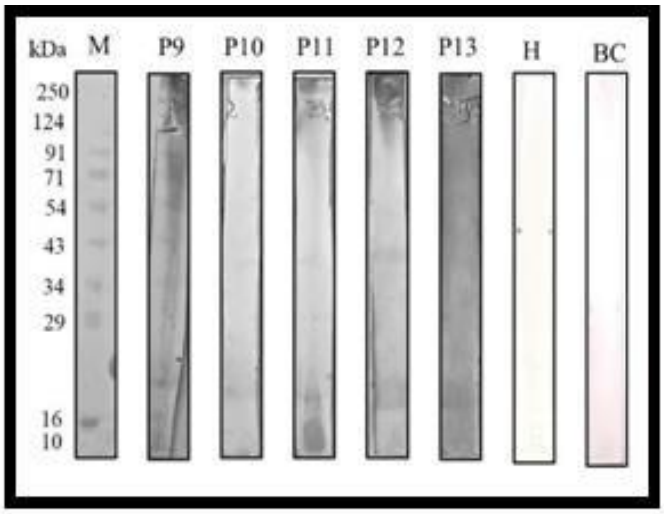

Figure 4. IgE specific Western blot set-2. M: MW ladder (Puregene, Genetix, New Delhi). P9-P13: IgE specific Western blot using sera from patients allergenic to *Aspergillus fumigatus*. H: Control, healthy serum, BC: Buffer control.

Figure 4 shows the membrane strips after Western blot of set-2 patient allergic sera towards *A. fumigatus*. In both cases all of them showed IgE reactivity with Asp f 3.

Recombinant allergen rAsp f 3 showing positive co-relation with a major fungal allergen

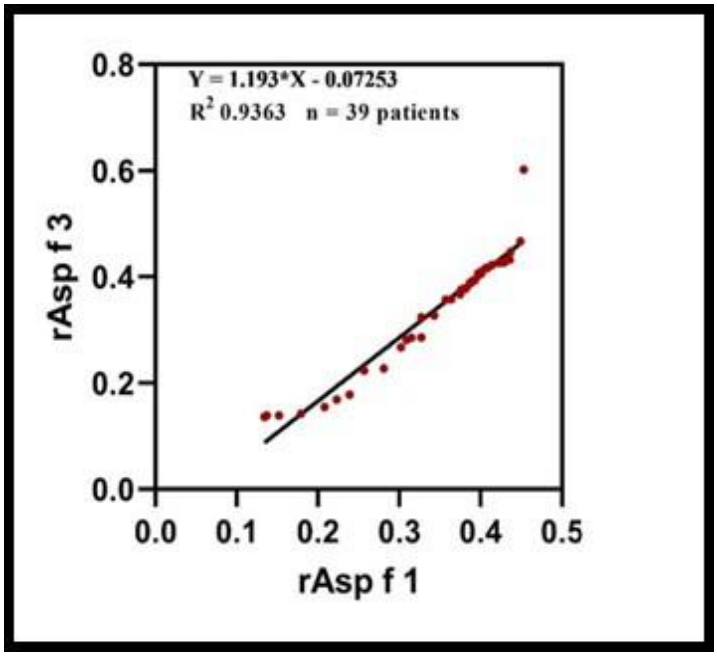

Figure 5. Linear regression analysis showing positive correlation between serological IgE-binding of Asp f3 and the major *Aspergillus* Allergen Asp f 1 within a population of 39 *Aspergillus*-positive patients.

From Figure 5 it was observed that rAsp f 3 and rAsp f 1 showed positive correlation. The R2 value was close to 1. As rAsp f 1 is a major fungal allergen already reported, the patient who have rAsp f 1 reactivity also have rAsp f 3 reactivity, showcasing as a major fungal allergen.

4. DISCUSSION

The prevalence of fungal allergies has been rising significantly, particularly in urbanized areas. However, when compared to pollen or food allergens, there is a scarcity of molecular studies focusing on fungal allergens. *Aspergillus fumigatus* is a common airborne fungus that can cause serious infections in immunocompromised individuals [15]. Environmental surveys indicate that ubiquitous spores of *A. fumigatus* are getting constantly inhaled by people all around. As a consequence, disease occurs predominantly in the lungs, although dissemination to virtually any organ may occur in the severely predisposed people.

The present study aims to understand the sero-prevalence of major fungal allergen Asp f 3 in Indian population. For this, the Asp f 3 cloned 'pBBL-TEV' vector was transformed into chemically competent *E. coli* cells. It was then subjected to IPTG induction to enhance the desired protein production. From here, desired Asp f 3 protein was purified using Ni-NTA based affinity chromatography to observe distinct bands of it in the SDS PAGE.

The IgE reactivity of the allergen was assessed in the sera of individuals of Indian origin. As the allergen was reported from other countries, the main aim to check the IgE reactivity using Western blot with the sera of Indian population. Then IgE reactivity was also assessed between rAsp f 1 and rAsp f 3, where rAsp f 1 is a major fungal allergen already reported, in this case we examined that in a population of 39 *Aspergillus* positive patients, a linear regression analysis revealed a positive correlation between serological IgE-binding to Asp f 3 and the major *Aspergillus* allergen, Asp f 1. The IgE- binding strength of Asp f 3 is comparable to that Asp f 1 which has already been established as a major allergen, suggesting that rAsp f 3 as a major fungal allergen in the context of Indian population [16].

5. CONCLUSION

Asp f 3 has already been established as a major allergen based on serology from non-Indian populations. Here we report a population-based data which shows sero-prevalence of Asp f 3 specific IgE in the sera of mold-allergic patients, suggesting its importance to be incorporated as diagnostic antigen. Also, as an ideal target for vaccine design.

6. ACKNOWLEDGEMENT

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