



Original Article

Prevalence of sensitization to *Aspergillus flavus* in patients with allergic bronchopulmonary aspergillosis

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Abstract

Aspergillus fumigatus is the most common *Aspergillus* species worldwide; however, *A. flavus* has also been shown to be prevalent in North India. Herein, we investigate the prevalence of sensitization to *A. flavus* in subjects with allergic bronchopulmonary aspergillosis (ABPA). We also evaluate the occurrence of allergic bronchopulmonary mycosis (ABPM) due to *A. flavus*. Treatment-naïve subjects with ABPA underwent sputum culture; and, skin testing, fungal-specific immunoglobulin E (IgE) and serum precipitation tests for *A. fumigatus* and *A. flavus*. Sensitization to *A. flavus* was diagnosed if any immunological test for *A. flavus* was positive in subjects with ABPA. ABPM was labelled as probable if sputum cultures grew *A. flavus* and *A. flavus*-specific IgE was greater than *A. fumigatus*-specific IgE; and, possible if only *A. flavus*-specific IgE was greater than *A. fumigatus*-specific IgE. Fifty-three subjects with a mean (SD) age of 34.2 (12.8) years were included. Sensitization to *A. flavus* was seen in 51 (96.2%) subjects, with overlap occurring in 49 (92.5%), 21 (39.6%), and 12 (22.6%) instances on fungal-specific IgE, skin prick test and precipitins, respectively. Sputum culture was positive in 18 (33.9%; *A. flavus* [$n = 12$], *A. fumigatus* [$n = 6$]) subjects. ABPM due to *A. flavus* was diagnosed in 16 (30.2%) subjects (10 probable, 6 possible). They were more likely to have high-attenuation mucus and a trend towards higher occurrence of sinusitis, compared to ABPA. We found a high occurrence of sensitization to *A. flavus* in subjects with ABPA. Subjects with

A. flavus-related ABPM had a higher likelihood of high-attenuation mucus and probability of sinusitis. More studies are required to confirm this observation.

Key words: fungal sensitization, *Aspergillus*, ABPA, asthma, ABPM, cystic fibrosis.

Introduction

Depending on the host immunity, the pulmonary disorders caused by fungi can be broadly classified into invasive, allergic and saprophytic. In the allergic spectrum, this interaction can range from allergic fungal sensitization to allergic mycosis.^{1–3} Allergic bronchopulmonary mycosis represents the severe end of allergy characterized by poorly controlled asthma, recurrent pulmonary opacities and bronchiectasis.⁴ The most common etiologic agent causing allergic bronchopulmonary mycosis is *Aspergillus fumigatus*. Conventionally, the disease is labeled as allergic bronchopulmonary aspergillosis (ABPA) when it is due to *A. fumigatus* and allergic bronchopulmonary mycosis (ABPM) when caused by fungi other than *A. fumigatus*.⁵ ABPA is a highly prevalent disorder,^{6,7} while the occurrence of ABPM is uncommon and mainly reported as sporadic cases.⁸

Although *A. fumigatus* is the most common species worldwide, an epidemiological survey demonstrated *A. flavus* also to be prevalent in the North-Indian environment,⁹ probably because of climatic factors.^{10,11} Additionally, *A. flavus* has been reported as the most common pathogen causing fungal sinusitis and endophthalmitis in the North Indian population.^{12–16} Interestingly, in several of our patients with ABPA, we have observed the growth of *A. flavus* in sputum cultures.¹⁷ It is possible that there is either co-sensitization or cross-sensitization between *A. flavus* and *A. fumigatus*;¹⁸ and *A. flavus* may be responsible for causing ABPM, hitherto unrecognized. In this study, we determine the prevalence of sensitization to *A. flavus* in patients with ABPA, and the occurrence of ABPM due to *A. flavus*. We also compare the clinical manifestations of ABPA and ABPM due to *A. flavus*.

Methods

This was a prospective observational study conducted between April 2015 and December 2016 in the Chest Clinic of this Institute. The study protocol was approved by the Institute Ethics Committee and a written informed consent was obtained from all the study participants.

Study participants

The cases included consecutive subjects with treatment-naïve ABPA. The diagnosis of ABPA was made if the patient met the modified ISHAM-ABPA working group

criteria.^{5,19} This includes all the following: (i) asthma; (ii) *A. fumigatus*-specific immunoglobulin E (IgE) >0.35 kUA/l; (iii) serum total IgE >1000 IU/ml; and presence of at least two of the following three components: (i) *A. fumigatus*-specific immunoglobulin G (IgG) >27 mgA/l; (ii) radiographic pulmonary opacities consistent with ABPA; (iii) peripheral blood eosinophil count >500 cells/ μ l.⁵ Subjects were further categorized as serological ABPA (ABPA-S) or ABPA-CB based on the absence or presence of bronchiectasis on high-resolution computed tomography (HRCT) of thorax, respectively.²⁰

Subjects with any of the following were excluded: (i) failure to provide informed consent; (ii) age >75 years; (iii) intake of systemic glucocorticoids or antifungal azoles for >3 weeks in the preceding 6 months; (iv) subjects receiving immunosuppressive drugs; and, (v) pregnancy.

Study procedure

All study participants meeting the inclusion criteria were subjected to detailed clinical history and physical examination. They also underwent the following investigations: *Aspergillus* skin test, *A. fumigatus* and *A. flavus* precipitins, *A. fumigatus* and *A. flavus*-specific IgE, sputum culture for fungus, total IgE and eosinophil count. Subjects complaining of nasal congestion, post-nasal drip, anosmia, headache and facial pain also underwent CT scan of the paranasal sinuses.

Aspergillus skin test

The test was carried out by injecting 0.2 ml of 100 protein nitrogen units (PNU)/ml of the *Aspergillus* (*A. fumigatus* and *A. flavus*) antigen (1 PNU = 0.00001 mg/ml) intradermally in the forearm, as previously described.²¹ The allergens used for performing the skin test were the crude extracts of *A. fumigatus* and *A. flavus* (Alcit India, Pvt. Ltd, New Delhi).

A. fumigatus-specific IgE levels

Levels were assayed using the M3 ImmunoCap with the commercial ImmunoCAP system (Phadia 100, Phadia, Uppsala, Sweden) utilizing the automated fluorescent enzyme immunoassay technology. The antigen used is an extract derived from the conidia and mycelia of *A. fumigatus* with a measuring range of an undiluted sample being 0.1–100 kUA/l. A value >0.35 kUA/l was considered as significant.²²

A. flavus-specific IgE

IgE was assayed using the M228 Immunocap with fluorescent enzyme immunoassay using the ImmunoCAP system. The antigen is an extract of *A. flavus* with a measuring range of 0.1–100 kUA/l (undiluted sample). A value >0.35 kUA/l was considered as significant.

A. fumigatus-specific IgG

IgG was assayed with fluorescent enzyme immunoassay using the ImmunoCAP system.²³

Total IgE levels

Levels were estimated with automated fluorescent enzyme immunoassay using the ImmunoCAP system.²⁴

Aspergillus precipitins

Precipitins were detected for *A. fumigatus* and *A. flavus* using the Ouchterlony gel double-diffusion technique as per the method described by Longbottom and Pepys.²³ The strength of the reaction was graded as 1+ to 3+. The allergens used were the extracts of *A. fumigatus* and *A. flavus*, prepared in the Department of Medical Mycology.

Sputum examination

Sputum was cultured for fungal growth using the following method. It was initially homogenized with N-acetyl-L-cysteine (NALC), treated with sodium citrate and neutralized with phosphate buffered saline. Subsequently, the sample was centrifuged, and the pellet was used for microscopy (calcofluor dye with potassium hydroxide) and culture (Sabouraud glucose agar medium). After mycelial growth, it was sub-cultured on malt extract agar and Czapek-Dox agar. The identification of *Aspergillus* species was done by morphological characteristics.

Sensitization to *A. flavus*

Sensitization was defined if the subject with ABPA had any positive test (specific IgE, precipitins or skin test) against *A. flavus*. Probable *A. flavus*-ABPM was diagnosed as: (1) growth of *A. flavus* on sputum culture AND *A. flavus*-specific IgE levels greater than *A. fumigatus*-specific IgE. Possible *A. flavus*-ABPM was diagnosed when *A. flavus*-specific IgE was more than *A. fumigatus*-specific IgE levels.

Study outcomes

We aimed to assess the: (i) presence of sensitization to *A. flavus* in subjects with ABPA; and, the (ii) occurrence of

ABPM due to *A. flavus*. We also compared the clinical manifestations of ABPA and ABPM due to *A. flavus*.

Statistical analysis

Data are presented in a descriptive fashion as mean (standard deviation [SD]) or number (percentage). The difference between continuous and categorical variables was analyzed using the Mann–Whitney *U* test and the Fischer exact test, respectively. A *P* value <.05 was considered as statistically significant.

Results

Fifty-three treatment-naïve ABPA subjects (56.6% males) with a mean (SD) age of 34.2 (12.8) years were enrolled (Table 1). The mean (SD) duration of asthma was 9.9 (7.1) years. A family history of atopy was present in six (11.3%) subjects. A history of expectoration of brown-black mucus was reported by 14 (26.4%) subjects. Bronchiectasis was encountered in 45 subjects, while eight were classified as ABPA-S. High-attenuation mucus was seen in 21 subjects. Nine (17%) subjects had one or more symptoms (nasal congestion, post-nasal drip, anosmia, headache, and facial pain) of sinusitis (Table 1). CT of the paranasal sinuses was performed in these subjects. Maxillary sinus was the most common sinus involved (Table 1). The most common finding on CT paranasal sinus was mucosal edema. High-attenuation mucus was seen in three subjects, while one subject had maxillary polyp. *A. flavus* was isolated in sputum in 12 (22.6%), while *A. fumigatus* was grown in six (11.3%) subjects.

Sensitization to *A. flavus*

Sensitization was seen in 51 (96.2%) subjects. Reactivity for fungus-specific IgE (49, 92.5%) was the most common followed by skin test (21, 39.6%) and precipitation reaction (12, 22.6%).

ABPM due to *A. flavus*

Of the 53 subjects with ABPA, 16 (30.2%) could also be labeled as ABPM due to *A. flavus* (Table 2). Ten and six subjects were labeled as probable and possible *A. flavus*-ABPM, respectively. On comparing subjects with ABPA to *A. flavus*-ABPM, there was no difference in age, sex distribution, and duration of asthma (Table 3). There was also no difference in the levels of total IgE and eosinophil counts. However, subjects with *A. flavus*-ABPM had significantly higher prevalence of high-attenuation mucus compared to

Table 1. Demographic and clinical profile of the study population ($n = 53$).

Variables	Values
Demography	
Male sex, n (%)	30 (56.6)
Age, years	34.2 ± 12.8
Height, centimeters	163.4 ± 10.5
Body weight, kilograms	58 ± 13.8
Duration of asthma, years	9.9 ± 7.1
Clinical features, n (%)	
Wheezing	50 (94.3)
Cough	52 (98.1)
Expectoration	30 (56.6)
Brown-black mucous plugs	14 (26.4)
Fever	5 (9.4)
Hemoptysis	15 (28.3)
Family history of atopy	6 (11.3)
Symptoms of sinusitis	
Nasal congestion	9 (17)
Anosmia	7 (13.2)
Post-nasal drip	9 (17)
Facial pain	8 (15.1)
Headache	9 (8.3)
Epistaxis	3 (5.7)
Immunological tests	
<i>A. fumigatus</i> -specific IgE, kUA/l	28.8 ± 21.5
<i>A. fumigatus</i> -specific IgG, mgA/l	73.8 ± 57.9
Total IgE, IU/ml	11152 ± 16300
Precipitins against <i>A. fumigatus</i> , n (%)	18 (34)
Skin test against <i>A. fumigatus</i> , mm	27.8 ± 14.3
Total eosinophil count, cell/ μ l	1126 ± 1468
Spirometry	
FEV1, liters	2.12 ± 1.0
FVC, liters	2.91 ± 1.1
CT PNS findings ($n = 9$)	
Polyp	1 (11.1)
HAM	3 (33.3)
Mucosal edema	8 (16.9)
Sinus involved	
Maxillary sinus	3 (33.3)
Frontal sinus	1 (11.1)
Ethmoidal sinus	0 (0)
Sphenoid sinus	1 (11.1)
Pan sinusitis	4 (44.4)
HRCT Thorax	
No. of segments with bronchiectasis	6.6 ± 4.7
HAM	21 (39.6)

All values are expressed as mean (standard deviation), unless specified otherwise

ABPA, allergic bronchopulmonary aspergillosis; ATT, anti tuberculosis therapy; CT PNS, computed tomography of paranasal sinus.

ABPA and a trend towards higher symptoms of chronic sinusitis (Table 3).

Discussion

The results of this study suggest that there is a high degree of sensitization to *A. flavus* in subjects diagnosed as ABPA. In the current study, almost one-third of the subjects with ABPA could be labelled as ABPM due to *A. flavus*. Subjects with ABPM due to *A. flavus* were more likely to have symptoms of sinusitis and higher prevalence of high-attenuation mucus on HRCT thorax.

The diagnosis of ABPA is currently made on the combination of clinical, radiological, and immunological findings.⁵ The antigens used most often for performing immunological tests in the diagnosis of ABPA are crude fungal extracts with potential for cross-reactivity.^{25,26} In the current study, serological tests for *A. fumigatus* were positive even amongst those whose sputum grew *A. flavus* and who had higher *A. flavus*-specific IgE than *A. fumigatus*-specific IgE. This phenomenon could occur either due to co-sensitization to both the *Aspergillus* species or due to immunological cross-reactivity. The presence of cross-reactivity can be discerned by performing qualitative IgE inhibition tests or by identifying the amino-acid sequence responsible for cross-reactivity. Unfortunately, the same could not be performed in the current study and is a major limitation of the study.²⁷ In a recent study, serological tests for *A. fumigatus* were positive in subjects whose sputum cultured other fungi such as *Aspergillus niger*, *Aspergillus terreus*, *Penicillium*, *Rhizopus oryzae*, and others.²⁸ However, even this study did not evaluate whether the polysensitization was due to co-sensitization or immunological cross-reactivity. While the growth of fungi on a single occasion may represent transient colonization without reflecting the long-term status of fungal colonization, the presence of high levels of fungal-specific IgE represents long-term exposure to the fungi. Whether fungal-specific IgE is more specific than fungal culture in the diagnosis of allergic mycosis remains unclear. In our study, subjects with ABPM who were culture-positive for *A. flavus* also had levels of *A. flavus*-specific IgE higher than *A. fumigatus*-specific IgE, suggesting that these could be ABPM due to *A. flavus*. The quantitative level of a patient's IgE against a particular antigen is at least partly indicative of the antigen that is more likely to be causing symptoms.^{18,29} On the other hand, skin test and *Aspergillus* precipitins were found to be unreliable in differentiating ABPM from ABPA, in the current study, similar to a previous study.²⁸

The true incidence of ABPM due to fungal species other than *A. fumigatus* remains unclear, with documentation mainly in the form of case reports or small case series.⁸

Table 2. Details of subjects with allergic bronchopulmonary mycosis (ABPM) due to *Aspergillus flavus*.

S no	Age, (years)	Sex	Sputum culture	<i>A. fumigatus</i> -specific IgE (kUA/l)	<i>A. flavus</i> -specific IgE (kUA/l)	PPT (<i>A. fumigatus</i>)	PPT (<i>A. flavus</i>)	Skin test (<i>A. fumigatus</i>)	Skin test (<i>A. flavus</i>)	TIGE (IU/ml)	TEC (cells/ μ l)	HAM on HRCT	Lobes with bronchiectasis on HRCT
Probable <i>A. flavus</i> -ABPM													
1	25	Female	<i>A. flavus</i>	9.91	15.2	+	+	21	25	15003	6720	Yes	5
2	23	Male	<i>A. flavus</i>	5.77	12.6	Negative	Negative	0	0	12936	563	No	5
3	51	Female	<i>A. flavus</i>	39.1	45	Negative	Negative	0	0	3210	200	Yes	5
4	38	Male	<i>A. flavus</i>	3	53.2	Negative	Negative	40	40	8409	1648	Yes	6
5	20	Male	<i>A. flavus</i>	23.9	29.1	Negative	Negative	51	0	3165	450	Yes	4
6	38	Male	<i>A. flavus</i>	25.9	44.4	++	+++	45	0	12077	866	No	4
7	27	Male	<i>A. flavus</i>	27	42	Negative	Negative	30	37	13903	251	Yes	5
8	56	Female	<i>A. flavus</i>	60.3	75.7	Negative	Negative	30	0	10331	222	Yes	2
9	38	Male	<i>A. flavus</i>	22.7	24.2	+	+	42	49	10570	953	Yes	5
10	44	Male	<i>A. flavus</i>	14.1	26.8	Negative	Negative	48	40	4462	1330	Yes	6
Possible <i>A. flavus</i> -ABPM													
11	30	Female	Sterile	32.6	35.5	+	+	21	30	39186	1584	Yes	4
12	28	Male	Sterile	13.7	17.4	Negative	Negative	18	0	5773	369	No	6
13	15	Male	Sterile	46.3	59.4	Negative	Negative	35	37	12176	1324	Yes	4
14	36	Female	Sterile	62.1	72.7	Negative	Negative	35	0	7134	700	Yes	6
15	39	Female	Sterile	13.5	27.8	Negative	Negative	0	0	1825	580	No	0
16	19	Male	Sterile	0.73	0.93	Negative	Negative	0	0	7367	724	No	0

FEV1, forced expiratory volume in one second; FVC, forced vital capacity; HAM, high attenuation mucus; HRCT, high resolution computed tomography; PPT, serum precipitins; TEC, total eosinophil count; TIGE: total IgE.

This is because most fungi are either mesophilic (unable to grow at body temperature, optimum growth occurring at 18–22°C) or thermophilic (can grow at body temperature but unable to grow below 20°C, so are not present in the environment) and rarely associated with human infections.³⁰ Thermotolerant fungi (especially *A. fumigatus*) can grow both in the environment and at body temperature and are thus associated with fungal allergy.³ *A. flavus* is unique in its ability to survive high ambient temperatures,¹⁰ with high prevalence in North India.^{12,13,16,17} Also, in a previous study of 80 subjects with ABPA, *A. flavus* was the most common (62%) fungi cultured in sputum.¹⁷

In the current study, subjects with ABPM had higher proportion of sinusitis and significantly higher prevalence of high-attenuation mucus on CT. Patients with *A. flavus*-associated ABPM have higher chances of sinusitis probably because of the larger size of the spores of *A. flavus*.³¹ The presence of high-attenuation mucus on CT has been shown to be associated with higher chances of relapse of ABPA.³² It is possible that ABPM due to *A. flavus* are associated with recurrent relapses and these subjects could have poor outcomes compared to ABPA.

Finally, our study has a few limitations. This was a small sample, single-center study and the results of this study should be confirmed in larger studies. We performed gel

double-diffusion technique to detect precipitins against *Aspergillus* species. Previously, it has been shown that IgG detected by enzyme immunoassay is more sensitive than the traditional test.^{23,33} It would also have been ideal if we had evaluated antibody responses against recombinant antigens of *A. fumigatus* and against other common fungi (including *Alternaria*, *Penicillium*, *Malassezia*, and others) to determine the extent of poly-sensitization; however, this could not be done due to financial constraints. We did not perform bronchoscopy to obtain respiratory samples because patients either did not consent or were considered as high-risk for bronchoscopy due to active pulmonary symptoms. Also, sputum was cultured only once because all patients were subsequently started on treatment. Finally, the allergen extracts used for skin testing, precipitin assay were different from the allergen extract used for IgE measurement, which might have also affected the results.

In conclusion, we found significant occurrence of sensitization to *A. flavus* in subjects with ABPA; and, high prevalence of ABPM due to *A. flavus* in North India. Subjects with ABPM due to *A. flavus* seem to have higher chances of sinus involvement and presence of high-attenuation mucus on CT chest. More studies are required to confirm our findings.

Table 3. Comparison of outcome parameters between ABPA and ABPM.

	ABPA (<i>n</i> = 37)	ABPM (<i>n</i> = 16)	<i>P</i> value
Positive fungal culture, <i>n</i> (%)	7 (18.9)	11 (68.7)	.002
<i>Aspergillus flavus</i>	1 (2.7)	11 (64.7)	
<i>Aspergillus fumigatus</i>	6 (16.2)	0 (0)	
Serological test			
<i>A. fumigatus</i> -specific IgE, kUA/l	30.4±22.5	25.0±18.9	.409
<i>A. flavus</i> -specific IgE, kUA/l	13.5±15.0	36.4±21.4	.0001
<i>A. fumigatus</i> -specific IgG, mgA/l	64.8±56.9	94.2±56.6	.092
Total IgE, IU/ml	11446±18762	10470±8689	.830
Precipitins against <i>A. fumigatus</i> , <i>n</i> (%)	14 (37.8)	4 (25.0)	.281
Precipitins against <i>A. flavus</i> , <i>n</i> (%)	9 (24.3)	4 (25.0)	1.0
Skin test against <i>A. fumigatus</i> , mm	27.9±13.4	27.6±16.8	.949
Skin test against <i>A. flavus</i> , mm	8.1±10.7	16.1±19.5	.606
Total eosinophil count, cell/μl	1113±1450	1155±1557	.924
Antigenic cross reactivity, <i>n</i> (%)			
Any cross-reactivity	35 (94.6)	16 (100)	0.343
Fungal-specific IgE	33 (89.2)	16 (100)	0.171
Skin testing	14 (37.8)	7 (43.8)	0.686
Precipitins	8 (21.6)	4 (25.0)	1.0
Symptoms of sinusitis	4 (10.8)	5 (31.3)	0.07
HRCT thorax,			
ABPA-S, <i>n</i> (%)	6 (16.2)	2 (12.5)	0.99
No. of segments with bronchiectasis	6.1 ± 4.9	8.3 ± 3.8	0.122
High-attenuation mucus, <i>n</i> (%)	10 (32.3)	11 (78.6)	0.004
Spirometry			
FEV1, liters	2.14 ± 1.11	2.05 ± 0.77	0.799
FVC, liters	2.93 ± 1.18	2.84 ± 0.82	0.781
FEV1: FVC ratio, percentage	68.9 ± 14.3	72.2 ± 11.3	0.444

All values are expressed as mean ± standard deviation, unless specified otherwise.

ABPA: allergic bronchopulmonary aspergillosis; ABPM: allergic bronchopulmonary mycosis; FEV1: forced expiratory volume in 1 second; FVC: forced vital capacity; HRCT: high resolution computed tomography.

Declaration of interest

RA: RA has received consultancy fees from Pulmatrix Inc. and has received funding from Cipla, India to carry out research work.

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The authors alone are responsible for the content and the writing of this paper.

Author contributions

ISS was involved in patient management and data collection, revised the manuscript for intellectual content.

HC performed the immunological tests and revised the manuscript.

SD was involved in patient management and revised the manuscript.

ANA was involved in patient management, statistical analysis, and revised the manuscript for intellectual content.

SB was involved in patient management and revised the manuscript.

MG was involved in patient management and revised the manuscript.

DB was involved in patient management and revised the manuscript.

AC was involved in patient management and revised the manuscript for intellectual content.

RA conceived of the idea, was involved in patient management, drafted and revised the manuscript for intellectual content.

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