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Research Article

Prevalence of Pulmonary Aspergillosis Among Chronic Lung Disease and Lung Cancer Patients with Reference to Clinico-Diagnostic Marker

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Abstract

Background: Invasive Aspergillosis is the most common opportunistic invasive mycoses. There is high prevalence of chronic lung diseases and lung cancers in our country.

Objectives: The present study was undertaken to determine the prevalence of Aspergillosis in patients suffering from chronic lung diseases and primary or metastatic lung cancer, to evaluate various clinico-diagnostic markers of pulmonary Aspergillosis and to study the antifungal susceptibility pattern of Aspergillus isolates.

Methods: The present study was conducted on 60 patients with chronic lung disease on whom bronchoscopy was performed. Histopathologic and radiological examination was done to identify carcinoma cell types and to categorize Aspergillosis types. Broncho alveolar lavage (BAL) fluids were collected for direct fungal examination, culture, Aspergillus polymerase chain reaction (PCR), and galactomannan (GM) detection and the antifungal susceptibility pattern of Aspergillus isolates was observed.

Results: Prevalence of Aspergillosis in patients of lung carcinoma was 32.5%. Aspergillus fumigatus was the predominant species isolated. Gutka chewing have significant role in predisposition to aspergillosis. Higher prevalence of galactomannan antigen was found in lung carcinoma as compared to other chronic lung diseases. 13.3% patients were of proven IPA, 15% of probable IPA, 6.6% belonged to possible IPA while 65% were of non IPA. Aspergillus DNA was detected in 41.6%. Isolates of Aspergillus spp. were most sensitive to Caspofungin, Amphotericin B, Ketoconazole and least to Itraconazole in that order according to Antifungal susceptibility using micro broth dilution method. Disk diffusion method of antifungal susceptibility showed highest resistance to Itraconazole and least to Caspofungin.

Conclusions: Higher prevalence of antigen was found in lung carcinoma as compared to other chronic lung diseases. This frequent association of Aspergillosis in lung carcinoma is quiet alarming, and we suggest that every patient with bronchogenic carcinoma should be screened for the presence of secondary Aspergillosis. Thus, early diagnosis of secondary Aspergillosis in such patients may be of great importance, because early antifungal treatment is associated with an improved outcome.

Keywords: *Aspergillosis; Galactomannan; Chronic Lung Disease; Lung cancer; Antifungal Susceptibility*

Introduction

The incidence of Invasive Pulmonary Aspergillosis (IPA) has increased during the past two decades and has become a major cause of mortality in severely immunocompromised patients. Major risk factors include neutropenia, hematopoietic stem-cell transplantation, prolonged and high dose corticosteroids therapy, hematological malignancies, cytotoxic therapy, advanced AIDS and Chronic Granulomatous Diseases [1]. Apart from these classical risk factors, other factors like critically ill patients admitted in intensive care units, patients with pre-existing lung disease (emphysema, chronic obstructive pulmonary disease, healed tuberculosis cavity), and patients with liver failure are also claimed to be associated with IA in developing countries [2]. Patients of primary or metastatic lung cancer are more prone

to *Aspergillus* implantation because of the malignant lung tissues and also because of long term steroids, anti-cancer chemotherapeutics and anti- bacterial therapy [3]. Once localized in the damaged lung, the mold grows and entails a very poor prognosis.

For years invasive Aspergillosis remained recalcitrant to facile or proper diagnosis. Isolation of an *Aspergillus spp*. from respiratory samples does not confirm it as the etiologic pathogen because airway colonization by *Aspergillus spp*. is a common feature in several chronic lung diseases. Repeated isolation of the identical *Aspergillus spp*. and detection of anti-*Aspergillus* antibodies and/or *Aspergillus* antigens in sera are needed to determine the isolate represents the etiologic agent of disease [4]. Conventional direct microscopy, histopathology, and culture methods are the only available techniques in most centers to diagnose IA due to the non-availability of galactomannan or beta-glucan tests assay in developing countries [5].

The Galactomannan antigen detection has been included in the Revised Criteria of EORTC/MSG for probable invasive Aspergillosis. Circulating antigen detection may contribute significantly to the diagnosis of invasive Aspergillosis; however, false-positive rates as high as 8% have been reported [3]. Therefore, now, the attention has turned to molecular detection methods, like polymerase chain reaction (PCR). However, the detection of nucleic acid is not included in the criteria as presently there are no validated or standardized methods [6]. The benefit of Polymerase chain reaction (PCR) is that while GM cannot identify infecting *Aspergillus spp*., PCR could be tailored to the species level and also possibly infer general antifungal susceptibility pattern [1]. Early initiation of antifungal therapy is critical in reducing the high mortality rate in these patients [7].

There is high prevalence of chronic lung diseases and lung cancers in India and the exact incidence of Pulmonary Aspergillosis in such patients is not known [8]. Early diagnosis of secondary Aspergillosis in such patients may be of great importance, because early antifungal treatment is associated with an improved outcome.

Keeping the above findings in view, the present study was undertaken to determine the prevalence of Aspergillosis in patients suffering from chronic lung diseases and primary or metastatic lung cancer, to evaluate various clinico-diagnostic markers of pulmonary Aspergillosis and to study the antifungal susceptibility pattern of *Aspergillus* isolates.

Materials and Methods

The present study was conducted on patients attending the outpatient department or admitted in the wards of T.B. and Respiratory Diseases, Jawaharlal Nehru Medical College and Hospital, Aligarh for a duration of one and half year. The study group comprised of 60 patients with chronic lung disease on whom bronchoscopy was performed. 25 age and sex matched healthy controls were included in the study with no evidence of any lung disease. A detailed clinical history, complete general and systemic physical examination was carried out with special reference to respiratory system. Informed written consent was taken prior to bronchoscopy from both study and control groups and the investigations were performed after approval from Institutional Ethics Committee.

Biopsy specimens for histopathology were collected by bronchial biopsy with the help of a fibre-optic bronchoscope (Olympus; BF Type TE2) and biopsy forceps (FB 20C-1). Broncho alveolar lavage was collected in sterile vials by fibre-optic bronchoscopy for direct microscopy, culture, galactomannan antigen (GM) detection, and PCR. Direct microscopy and cultures were performed immediately, whereas Broncho alveolar lavage (BAL) specimens for performing detection and PCR were stored at -20°C till they were tested. About 5 ml blood was collected by vein-puncture with all aseptic precautions for GM and PCR. Specimens collected were transported immediately to the laboratory for processing. Serum was separated and stored at -20°C until they were further processed.

Processing of BAL

BAL sample was homogenized using vortex and subjected to following laboratory procedures.

Direct Microscopy: Direct mount and lactophenol cotton blue (LPCB) mount were prepared according to the standard procedure to look for fungal elements like fungal hyphae, vesicle, and spores of Aspergillus species.

Culture: BAL was streaked on two sets of Sabouraud Dextrose Agar (SDA) plain and SDA containing chloramphenicol (0.05 mg/ml) and on two sets of Czapek dox agar. One set of both the media was incubated at 25°C and the other at 37°C. The fungal isolates were confirmed and characterized according to the standard techniques [9].

Antifungal Susceptibility Testing

Determining the Minimum Inhibitory Concentration: Antifungal susceptibility against Amphotericin B (AMB), Itraconazole (ICZ), and Caspofungin (CAS) was determined by using the broth microdilution method of Espinel-Ingroff., *et al*. [10] with some modifications. Briefly, the medium that was used for sensitivity testing was RPMI-1640 buffered with 0.165 Mol/L 3-(N-morpholino) propane sulfonic acid (Hi-Media Laboratories). The solvent that was used for the inoculums preparation and for making dilutions of the drugs was dimethyl sulfoxide. The dilution series were in the range from 16 µg/mL to 0.0313 µg/mL for all drugs. Each microdilution well was inoculated with 100 µL of double-diluted drug concentration and 100 µL of double-diluted (5×104 colony-forming units/mL) conidial inoculum suspensions.

The last well of each row served as the growth control. One well of the microdilution plate that contained un-inoculated, drug-free medium served as a sterility control. *A. flavus* (American Type Culture Collection [ATCC] 204304 and *A. fumigatus* ATCC 204305 were included as standard control strains. The microplates were incubated at 35°C without agitation and were examined after 48 hours to determine the minimum inhibitory concentration (MIC).

Disc Diffusion Method: Antifungal susceptibility by disk diffusion was done on non-supplemented MHA according to the method described in CLSI M 51-A document (CLSI-2010). In brief entire surface of MHA was inoculated with non-toxic cotton swab with the undiluted mold stock inoculum suspension. Disks of AMB (10µg, prepared In-house); VCZ (10 µg, Hi-media); CAS (5 µg, In-house) were placed onto the surface of each of the inoculated MHA plate. The plates were incubated at 350C and were read after 24h and 48h. Slight trailing around the zone edge or hyphal element extending into the inhibition zone was ignored when testing triazoles, but not for AMB. Micro-colonies or trailing growth within a well-defined zone of inhibition was ignored when testing CAS. Zone diameter were interpreted in accordance with CLSI M51-A. In brief for AMB isolates were considered as WT at zone size ≥ 15 mm. For ICZ and CAS, isolates were considered as WT at zone size ≥ 17 mm (CLSI-2010).

Detection of Aspergillus Antigen (Galactomannan) in Sera and BAL Samples

Galactomannan antigen was detected in BAL and serum samples using PLATELIAT^{M} *Aspergillus* Ag ELISA kit (Bio Rad, Marnes-la-Coquette, France). Test was performed as per the manufacturer's instructions and Platelia EIA index cut-off value of ≥ 0.50 was considered positive.

Detection of Aspergillus-DNA in BAL-polymerase chain reaction (PCR)

Template preparation for PCR: DNA extraction was done according to the method described by Lee & Taylor [11] with minor modifications. Briefly to 100µl of the homogenized BAL, 400 µl of lysis buffer containing 50 mMTris-HCl (pH 7.2), 50 Mm EDTA, 3% SDS, and 1% β-mercaptoethanol was added. The mixture was vortexed for 15 secs, incubated at 65°C or 1 hr and then boiled for 10 min. An equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) was added to the mixture and vortexed for 10 sec. Mixture centrifuged at 14,000for 15 min and to the aqueous phase, 10 µl of 3M Sodium Acetate (pH 5.2) and 5.4 volume of the isopropanol were added. The tubes were inverted to mix gently, and the DNA was precipitated at -20°C for 1h. The tubes were again micro centrifuged at 14,000 rpm for 10 min and the supernatant was decanted. The DNA-pellets were washed with 70% ethanol, dried and resuspended in 25 µl of sterile Millipore water. Ten µl of DNA template was used in PCR reaction.

PCR assay: The primers used in the study (5'- GAA AGG TCA GGT GTT CGA GTC AC 3'and 5'- CTT TGG TTG CGG GTT TAG GGA TT 3') amplified 135 base pairs (bp) of *A. fumigatus*, 118 bp of *A. flavus*, and 180 bp of *A. niger* [12]. All PCR reactions were carried out with 50 µl reaction volume in 0.2 ml thin wall polypropylene tubes (Axygen, Inc, USA).

The reagents added to the PCR tubes were 22.5 µl of SuperMix (AccuPrime™ *Pfx*SuperMix, Invitrogen life technologies, California) containing 22 U/ml *Thermococcus* species KOD thermo stable polymerase complexed with anti-KOD antibodies, 66 mM Tris-SO4 (pH 8.4), 30.8 mM (NH4)2SO4,11 mM KCl, 1.1 mM MgSO4, 330 µM dNTPs, AccuPrime™ proteins, stabilizers,10 pM of each primer (200 nM each), 10 µl of DNA template and sterile Millipore water to make up the volume to 50 µl. The PCR cycle consisted of an initial denaturation step at 95°C for 5 min followed by 40 cycles of 94°C for 30 secs, 72°C for 1 min, and final elongation at 72° C for 10 min. Two-microliter of PCR products was analysed by gel electrophoresis with 2% agarose containing SYBR Safe DNA gel stain (Invitrogen life technologies, California). A 100 bp ladder was used as molecular marker (Trackit™, Invitrogen life technologies, California).

Histopathological Study of Biopsy Tissue

Biopsied tissue was studied for the confirmation of clinical diagnosis and fungal invasion by hematoxylin and eosin staining [13], and periodic acid Schiff staining (PAS), respectively [14].

Clinical Classification of Aspergillosis

All patients were categorized into different categories based on the criteria of the European organization for research and treatment for cancer/mycoses study group (EORTC/MSG) as defined by Ben de Pauw., *et al*. [6]. These categories are: (1) proven IFD, (2) probable IFD, and (3) possible IFD.

Statistical Analysis

Sensitivities, specificities of results of non-validated tests, i.e., PCR, ELISA, Culture and Conventional microscopy for Proven IPA, probable IPA, possible IPA, and non-IPA, respectively, were determined for the entire dataset. For other variables, statistical analysis was performed by using the chi-square test, and P values < 0.05 were considered statistically significant. For statistical analysis, we used SPSS 22.0 software (SPSS, Inc., Chicago, Ill).

Results

In this study, age of the patient showed that the cases of chronic lung diseases increased with age up to 61 - 70 years where the maximum cases were reported and then the occurrence of disease declined. Majority (73.3%) of patients of chronic lung diseases especially lung carcinoma was found in the age group of 51 to 70 years. Mean age of cases was 55.3 years. Male to female ratio in our study was 11:1. Farmers comprised the major occupational group (41.6%), followed by laborers (16.6%). Majority of farmers were involved in farming of wheat, maize, bajra and jowar in the decreasing frequency. Laborers were second in majority (16.6%).

Majority of patients in our study group had malignancy of lung with 66.6% of cases having primary lung cancer while 26.6% cases had secondaries in the lung. Rests of the patients (6.5%) were of other chronic lung diseases.

Lung Diseases	No. of cases	Percentage (%)
Lung Carcinoma	40	66.6
Secondaries in lung	16	26.6
COPD	1	1.6
Pulmonary tuberculosis	2	3.3
Sarcoidosis	1	1.6
Total Cases	60	100

Table 1: *Distribution of patients according to clinical disease.*

In histopathology of biopsy tissue, predominant type of carcinoma was found to be squamous cell carcinoma (47.5%) while 37.5% patients were diagnosed with adenocarcinoma. *Aspergillus spp*. was isolated from BAL in 18 (30%) of the total cases. The age of patients

with pulmonary Aspergillosis ranged from 31 - 70 years with mean age of 59.61 years. Out of total cases positive for culture 16 (88.8%) were males and 2 (11.1%) were females. In our study, prevalence of Aspergillosis in patients of lung carcinoma was 32.5%. *Aspergillus fumigatus* was the predominant species isolated (50%) in BAL. *A. flavus* contributed 38.9%, *A. niger* 5.5% and *A. nidulans* 5.5% of all the *Aspergillus species* isolated.

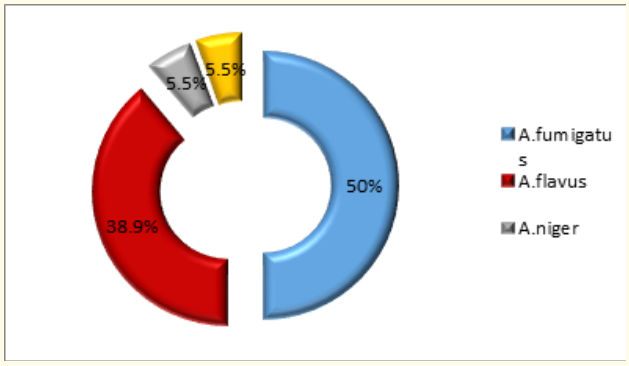

Figure 1: *Relative number of different Aspergillus species isolated in BAL of patients (n=60).*

When culture positivity for Aspergillus was compared in smokers and non- smokers, though smokers were found to have slightly higher percentage of culture positive cases (36.1%) as compared to non-smokers (33.3%) in our study, the finding was not statistically significant (p = 0.65). However, gutka chewing was found to be positively associated with Aspergillus infection (p = 0.014).

Addiction	Culture Positive for Aspergillus species (n=18)	Culture Negative for Aspergillus species (n=42)	Total cases	Percentage of Culture Positives (%)	Odds ratio (95%CI)
Smokers	9	36	45	20	1 (0.23-4.30)
Non smokers	3	12	15	20	
Gutka chewing present	6	25	31	19.3	0
No gutka chewing	0	29	29	0	

Table 2: *Correlation of culture positivity with smoking and gutka chewing habits.*

We studied the isolation of various *Aspergillus spp*. in relation to occupation. 9 (36%) of the farmers were positive for *Aspergillus* culture in their BAL fluid. 50% of the factory workers who were employed in other occupations but they were exposed to food grains in some form like loading grains in field, employed in food packing factory and flour mill showed the growth of Aspergillus in their BAL samples.

We tested all BAL and serum samples for galactomannan antigen as well. Antigen was detected in 38.3% of samples. Both BAL and Sera were positive for GM antigen in 23.3% of cases while in 15% of the patients' galactomannan was positive only in BAL and their sera were negative for the antigen. No case showed GM antigen in serum alone. On correlating galactomannan antigen with smoking and gutka chewing habits, we found 44.4% of the smokers were positive for galactomannan antigen. 20% of patients addicted to gutka chewing only were positive for the antigen. Patients with no history of addiction were negative for galactomannan antigen. Overall, GM assay showed a sensitivity of 100% and a specificity of 86.4%.

Among patients of lung carcinoma galactomannan antigen was positive in 37.5% of BAL and 25% of serum samples. Here again in 18.7% patients only BAL showed the presence of galactomannan antigen. In patients with secondary's in lung 25% were positive for culture and 37.5% had galactomannan antigen positive. In cases of tuberculosis 1 (50%) had culture positive while 1 (50%) of the 2 was positive for galactomannan antigen in BAL fluid only. None of the patient of COPD was positive for culture or galactomannan.

Disease	Culture for Aspergillus		Galactomannan antigen			
	Positive	Negative	Total no. of cases positive for galactomannan antigen (%)	Only BAL positive (%)	Both BAL and serum positive (%)	Both BAL and serum negative (%)
Lung carcinoma (n = 40)	13 (32.5)	27 (67.5)	15 (37.5)	5 (12.5)	10 (25)	25 (62.5)
Secondary's in lung (n = 16)	4 (25)	12 (75)	6 (37.5)	3 (18.7)	4 (25)	10 (62.5)
Tuberculosis (n = 2)	1 (50)	1 (50)	1 (50)	0	0	1 (50)
COPD (n = 1)	0	1 (100)	0	0	0	1 (100)
Sarcoidosis (n = 1)	0	1 (100)	1 (100)	1 (100)	0	0
Total (n=60)	18 (30)	42 (70)	23 (38.3)	9 (15)	14 (23.3)	37 (61.6)

Table 3: *Culture findings and galactomannan antigen in relation to clinical diseases.*

In our study, culture examination for Aspergillus was positive in 30% of cases which was less as compared to galactomannan antigen in both (38.3%) BAL and sera. 18 patients showed the growth of Aspergillus on culture while 23 patients were positive for galactomannan antigen. Hence culture positivity was seen in 78.2% of antigen positive patients.

On categorizing the patients according to criteria of the European Organization for Research and Treatment of Cancer/Mycoses Study Group (EORTC/MSG) as described by Ben De Pauw., et al. found that 8(13.3%) patients were of proven invasive pulmonary Aspergillosis (IPA), 9(15%) of probable IPA, 4(6.6%) belonged to possible IPA while 39(65%) were of non IPA.

Aspergillosis type		Direct microscopyPositivity/ HPE positive	Culture for aspergillosis-Positivity BAL only	Samples positive for galactomannan antigen			CT scan findings positive for Aspergillus
				Serum only	Both BAL and serum		
IPA (n=21)	Proven IPA (n = 8)	7	8	2	0	6	8
	Probable IPA (n = 9)	5	9	2	0	7	6
	Possible IPA (n = 4)	0	1	3	0	1	2
Non IPA (n=39)		0	0	2	0	0	3
Total (n=60)		12	18	9	0	14	19

Table 4: *Classification of patients according to Aspergillosis type.*

**HPE- Histo-pathological examination.*

We studied populations various clinical signs and risk factors for all the patients in our study that helped us in categorizing study population. Amongst the clinical signs, cough and chest pain was found in most of the cases of IPA followed by dyspnea, fever and hemoptysis. Major risk factor was found to be neutropenia (polymorph nuclear neutrophil count, < 0.5 G/L), broad-spectrum antibacterial agents and corticosteroids, cytotoxic chemotherapy and other immunosuppressive therapies (e.g., cyclosporine, tacrolimus, methotrexate, cyclophosphamide and azathioprine etc.

Characteristics	Proven IPA (n=8)	Probable IPA (n=9)	Possible IPA (n=4)
Clinical sign:			
Fever	5 (62.5)	7 (77.7)	2(50)
Dyspnea	6 (75)	5 (55.5)	1 (25)
Cough	7 (87.5)	8 (88.9)	1 (25)
Chest pain	8 (87.5)	6 (66.6)	2 (50)
Hemoptysis	3 (37.5)	2 (22.2)	0
Neutropenia			
(<500 neutrophils/mm^3)	8 (100)	7 (77.8)	3 (75)
Clinical disease:			
Lung carcinoma(n=40)	6 (75)	5 (55.5)	2 (50)
Tuberculosis(n=2)	0	0	1 (25)
COPD(n=1)	0	0	0
Sarcoidosis (n=1)	0	1 (11.1)	0
Drugs:			
Antibiotics	5 (62.5)	4 (44.4)	2 (50)
Corticosteroids	8 (100)	6 (66.7)	1 (25)
Immuno suppressants	5 (62.5)	4 (44.4)	1 (25)
Chemotherapeutics	6 (75)	3 (33.3)	1 (25)
Thoracic CT sign:			
Dense well circumscribed lesion(s) ± Halo sign	5 (62.5)	4 (44.4)	1 (25)
Air-crescent sign	4 (80)	5 (55.5)	1 (25)
Cavity	6 (75)	2 (22.2)	0

Table 5: *Comparison of clinical and biological features of Proven, Probable, and Possible IPA*

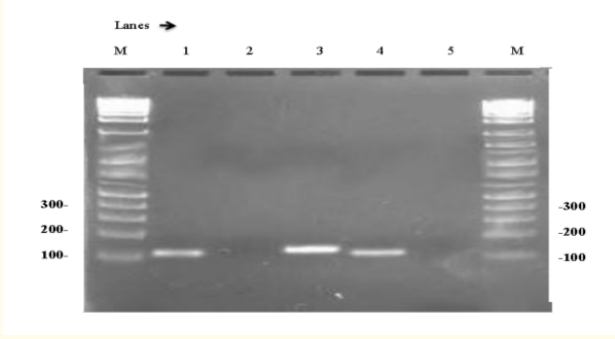

Figure 2: *Electrophoretogram of bronchoalveolar lavage polymerase chain reaction on a 2% agarose gel shows amplicons of 135 base pairs (bp) of Aspergillusfumigatus and 118 bp of A. flavus.*

Lane 1 indicates positive control. Lane 2 indicates negative control. Lane 3& 4 indicate positive samples.M indicates molecular weight marker.

PCR was performed and the findings for *Aspergillus spp*. in relation to clinical disease were seen. It was seen that 20/40(50%) cases of lung carcinoma and 4/16(25%) cases of secondaries in lung were detected by PCR for *Aspergillus spp*. Aspergillus DNA was detected in 38.3% (23/60) cases. PCR was positive in all the proven, probable IPA cases, while only 2/4 possible cases of IPA and 4/39(10.2%) cases of non IPA were PCR positive.

All the control patients had negative PCR tests. Two main *Aspergillus species* isolated by PCR in all the categories of IPA were *A. fumigatus* and *A. flavus. A. fumigatus* was found in 6/8 (75%) cases of proven IPA, 5/9 (55.5%) cases of probable IPA, ¼ (25%) cases of possible IPA and 2/39 (5.1%) non-IPA patients. *A. flavus* was found in 2/8 (25%) cases of proven IPA, 4/9 (44.4%) cases of probable IPA, 1/4 (25%) cases of possible IPA and 0/39 Non-IPA patients. 1 (2.5%) amplicon of *A. niger* and 1 (2.5%) of *A. nidulans* was detected in Non-IPA patients. Our study showed that PCR has an overall sensitivity of 100% and a specificity of 81.3%.

To compare the diagnostic value of the various tests, we divided the Proven IPA and Probable IPA cases as the one diseased IPA group and the possible IPA and Non IPA as the non-diseased group. We found that PCR and GM assay were able to significantly differentiate between Invasive Aspergillosis and *Aspergillus* colonization. Because false-positive results have been reported with PCR [15], we believe that combining PCR with serologic tests may help to confirm the diagnosis of Aspergillosis.

We tested antifungal susceptibility using Broth microdilution method (CLSI M-38-A2). MIC ranges for Amphotericin B, Itraconazole, Caspofungin in all the 18 Aspergillus isolates was identified. High MIC of >2µg/ml for Amphotericin B was found in *A. flavus* (14.2%) and *A. niger* (100%). MIC of > 8µg/ml for Itraconazole was found in *A. fumigatus* (11.1%). MEC for Caspofungin was found to be sensitive (< 1 µg/ml) for all the *Aspergillus* isolates.

CLSI in year 2010 published disk diffusion method of antifungal susceptibility of non-dermatophyte filamentous fungi (CLSI M51-A). It was observed that, out of a total of 18 Aspergillus isolates, 14 (77.8%) isolates were sensitive to Amphotericin B, 13 (72.2%) to Itraconazole, while 18 (100%) were sensitive to Caspofungin.

To find out the robustness of the diagnostic values of the tests (performed) to discriminate the pulmonary invasion and airway colonization, we analyzed the data assuming Proven IPA and Probable IPA as diseased group and possible IPA and non IPA as non-diseased group. We found, all the patients with proven IPA and probable IPA had positive PCR, GM serology and culture compared to control patients ($p < 0.0001$). However, microscopy as a method to differentiate between Aspergillus invasion and colonization was found to be statistically non-significant.

Test Used	Invasive Aspergillosis	Aspergillus colonization	Chi square χ^2	p value
PCR + PCR-	17 0	8 35	33.21	<0.0001
GM Ag ELISA+ GM Ag ELISA-	17 0	6 37	38.16	<0.0001
CULTURE+ CULTURE-	17 0	1 42	55.35	<0.0001
MICROSCOPY+ MICROSCOPY-	12 5	0 43	-	NS

Table 6: *Evaluation of the discriminatory power of PCR, serology, culture and microscopy between pulmonary invasion (True disease) and airway colonization (no disease) [Assuming proven and probable IPA representing true disease and possible and non-IPA representing no disease].*

**PCR-Polymerase chain reaction; GM Ag ELISA-Galactomannan antigen enzyme linked immunosorbent assay; NS-Non Significant.*

Discussion

Invasive Aspergillosis (IA) is a disease of concern and the risk factors for development of IA in developing countries appear to be similar to the developed world, though certain differences were observed due to local epidemiology. Since reliable diagnosis of invasive aspergillosis at an early stage is often extremely challenging and early effective therapy is critical for successful control of established disease, most practitioners tend to rely on generalized antifungal prophylaxis and empirical therapy.

In our study, majority of patients 23/60 (38.3%) were in the age group of 61 - 70 years. Mean age of cases was 55.3 years and male to female ratio in this study was 11:1. A. Cornillet., *et al*. [16] also reported the mean age of 52 years in their study on pulmonary Aspergillosis.

Farmers comprised the major occupational group (41.6%). Jan E Zejda., *et al*. [17] reported that agricultural work environment holds the potential for exposure to many respiratory biohazards. Majority of patients had malignancy of lung with 66.6% of cases having primary lung cancer while 26.6% cases had secondaries in the lung. Shahid., *et al*. [5] also reported in their study on chronic lung diseases for invasive aspergillosis that the majority of cases (47.7%) were of bronchogenic carcinoma followed by other chronic lung diseases.

75% of study patients were smokers and of them 80% of the cases of lung carcinoma had history of smoking. Patients who were addicted to both smoking as well as gutka chewing also had an appreciable prevalence of lung carcinoma (30%). 16.6 % of patients who were non-smokers and addicted to gutka chewing alone also developed lung carcinoma. In a survey by D. Behera [18], 90% lung cancers were attributable to tobacco smoking.

The main presenting features of patients suffering from chronic respiratory diseases were cough (76.7%), followed by shortness of breath (61.6%). In a study conducted by Sana., *et al*. [19] on patients of bronchogenic carcinoma had reported similar symptoms like cough (73%) and shortness of breath (51.1%) as the main presentation of the patients.

Predominant type of carcinoma was squamous cell carcinoma (47.5%) while 37.5% patients were diagnosed with adenocarcinoma. Travis (2002) also reported that the prevalence of 80% of squamous cell carcinoma accounts for 25% of the cases of lung carcinoma.

Aspergillus spp. was isolated from BAL in 18 (30%) of the cases. The age of patients with pulmonary Aspergillosis ranged from 31-70 years with mean age of 59.61 years. *Aspergillus fumigatus* was the predominant species isolated (50%) in BAL. *A. flavus* contributed 38.9%, A. niger 5.5% and *A. nidulans* 5.5% of all the *Aspergillus spp*. isolated. Shahid., *et al*. [5] reported 14.7% prevalence of Aspergillus in BAL in patients with chronic lung diseases while A. Cornillet reported BAL culture positivity of 68.6% for Aspergillus. Though there is no definite trend but it seems quite obvious that the prevalence of *Aspergillus* infections of the lungs is on the rise.

Culture positivity for Aspergillosis in smokers was found to have slightly higher percentage of culture positive cases (36.1%) as compared to non-smokers (33.3%). However, gutka chewing was found to be positively associated with *Aspergillus* infection. 36% of the farmers were positive for *Aspergillus* culture in their BAL fluid ($p = 0.014$). Though direct evidence of gutka/snuff addiction with pulmonary Aspergillosis could not be found but in a study conducted by Varma, it has been demonstrated that *Aspergilli* are the dominating microflora of chewing tobacco leaves in storage.

GM antigen was detected in 38.3% of samples. Both BAL and sera were positive for GM antigen in 23.3% of cases while in 15% of the patients' galactomannan was positive only in BAL and their sera were negative for the antigen. Galactomannan antigen was found positive in 44.4% of the smokers and 20% of patients addicted only to gutka chewing were positive.

Culture examination for Aspergillus was positive in 30% of cases which was less as compared to galactomannan antigen in both (38.3%) BAL and sera.

Among patients of lung carcinoma galactomannan antigen was positive in 37.5% of BAL and 25% of serum samples. This is similar to the 36.2% galactomannan positivity in BAL of lung carcinoma patients as reported by M Shahid., *et al*. [3]. Patients with secondaries in

lung 25% were positive for culture and 37.5% had galactomannan antigen positive. In cases of tuberculosis 1 (50%) had culture positive while 1 (50%) of the 2 was positive for galactomannan antigen in BAL fluid only. None of the patient of COPD was positive for culture or galactomannan.

Higher prevalence of antigen was found in lung carcinoma as compared to other chronic lung diseases. This frequent association of Aspergillosis in lung carcinoma is quiet alarming, and we suggest that every patient with bronchogenic carcinoma should be screened for the presence of secondary Aspergillosis.

All patients were categorized into 4 categories as proven IPA, probable IPA, possible IPA and non-IPA.8 (13.3%) patients were of proven invasive pulmonary Aspergillosis (IPA), 9 (15%) of probable IPA, 4 (6.6%) belonged to possible IPA while 39 (65%) were of non IPA.

In a study conducted by Sana., *et al*. [19] they found, 20% patients of proven IPA, 32.5% of probable IPA and 17.5% of possible IPA and 30% of their patients did not meet any of the criteria for IPA.

All the cases of proven IPA were positive for BAL culture and 5 (8.3%) of them showed fungal elements for Aspergillus on direct microscopy and 2 (3.3%) showed histopathological findings of Aspergillus. All these patients were also positive for galactomannan antigen (GM). Also, all the cases showed radiological findings positive for pulmonary Aspergillosis.

In case of probable IPA all the 9 patients were positive on BAL culture, 5 showed fungal elements on direct microscopy, 2 of them had galactomannan antigen in BAL and 7 had antigen in both BAL and serum. Radiological findings were positive only in 6 out of 9 patients.

In case of possible IPA 1 of the 4 patients were positive for BAL culture. 3 cases were positive for GM antigen in the BAL and 1 had antigen positive in both BAL and serum. 2 showed radiology findings positive. Among non IPA none of the 39 cases were positive for *Aspergillus* culture nor showed ant fungal elements on direct microscopy. 2 patients had antigen in BAL while none had antigen in both BAL and serum while 3 of the cases showed radiological findings conclusive of Aspergillosis.

Various clinical signs and risk factors for all the patients were studied. Amongst the clinical signs, cough and chest pain was found in most of cases of IPA followed by dyspnea, fever and hemoptysis. High-resolution Thoracic CT-scan was performed and the principal findings were dense circumscribed lesion(s) ± halo sign, air-crescent sign, and cavity. Neutropenia, broad-spectrum antibacterial agents, corticosteroids, cytotoxic chemotherapy and other immunosuppressive therapies were the major associated risk factors.

PCR for *Aspergillus spp*. was done and correlated in relation to clinical disease. 50% cases of lung carcinoma and 25% cases of secondaries in lung were detected by PCR for Aspergillosis. Neither of the 2 cases of COPD and sarcoidosis each was positive for Aspergillosis by PCR. However, 50% of tuberculosis was Aspergillus PCR positive.

Aspergillus DNA was detected in 41.6% cases. Two main *Aspergillus spp*. isolated by PCR in all the categories of IPA were *A. fumigatus* and *A. flavus*, *A. fumigatus* was found in 75% cases of proven IPA, 55.5% cases of probable IPA, 25% cases of possible IPA and 5.1% non-IPA patients. *A. flavus* was found in 25% cases of proven IPA, 44.4% cases of probable IPA, 25% cases of possible IPA and 0 in non-IPA patients. 2.5% amplicons of *A. niger* and 2.5% of *A. nidulans* was detected in non-IPA patients.

The role of BAL-PCR in the diagnosis of IPA as compared with the conventional methods was evaluated. Microscopy had low sensitivity of 70.5%. Culture had highest specificity of 97.6%. GM assay showed a sensitivity of 100% and a specificity of 86.4%. PCR has an overall sensitivity of 100% and a specificity of 81.3%.

The diagnostic performance of PCR was similar to that of GM in BAL fluid in proven, probable IPA cases and possible IPA cases. We believe that the diagnostic performance of BAL PCR is good and comparable to that of GM in BAL. To establish a firmer diagnosis, GM ELISA should be combined with BAL PCR in the laboratories where facilities are available. The results can be forwarded to the physicians the very next day.

Antifungal susceptibility using broth microdilution method was seen for Amphotericin B, Itraconazole and Caspofungin. MIC range of Amphotericin B for *A. fumigatus* was within sensitive range (≤ 2µg/ml) for all the isolates. *A. flavus* had high MIC of 4µg/ml in 14.2% isolates. MIC of 1 isolate of *A. niger* was high of 4µg/ml and 1 isolate of *A. nidulans* was sensitive with low MIC.

For Itraconazole, in case of *A. fumigatus* only 1/9(11.1%) isolates had high MIC of 16µg/ml. MIC ranges for *A. flavus* was in sensitive range except for the high MIC of 8µg/ml in 2/7(28.5%) isolates. MIC of *A. niger* and *A. nidulans* was in the sensitive range.

MEC ranges for Caspofungin of all *Aspergillus spp*. isolated was within sensitive range with MEC of ≤ 1µg/ml. Resistance to Caspofungin in Aspergillus had not been reported from India. This could be because Caspofungin is still the least used antifungal in India.

Disk diffusion method of antifungal susceptibility test when done showed 14 (77.8%) isolates were sensitive to Amphotericin B, 13 (72.2%) to Itraconazole, 15 (83.3%) to Ketoconazole while 18 (100%) were sensitive to Caspofungin.

Resistance to Amphotericin B was observed in 22.2% of *A. fumigatus*, 14.2% of *A. flavus* and 100% of *A. niger*. Resistance to Itraconazole was seen in 22.2% of *A. fumigatus*, 28.5% of *A. flavus* and 100% of *A. niger*. Resistance to Ketoconazole was seen in 11.1% of *A. fumigatus*, 14.2% of *A. flavus* and 100% of *A. niger*. No resistance was observed against Caspofungin against any species of *Aspergillus*.

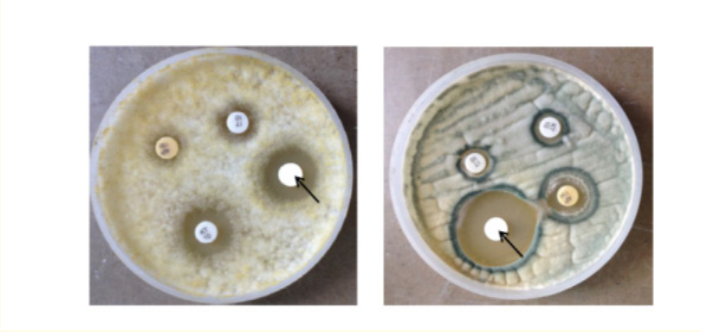

Figure 3: *Antifungal susceptibility testing by Disc Diffusion method for A. flavus.*

Figure 4: *Antifungal susceptibility testing by Disc Diffusion method for A. fumigatus.*

Figure 5: *Antifungal susceptibility testing by Disc Diffusion method for A. niger*

The recently expanded antifungal armamentarium offers potential for more effective and less toxic therapy for this lethal infection. Early therapy is critical for a successful outcome, but the diagnosis remains difficult and knowledge of the clinical presentation and risk factors can lead to a heightened suspicion enabling earlier diagnosis.

Several factors influencing the clinical outcome of patients were also analysed. Out of the total 60 patients, only 56.7% patients could be followed. 26 (43.3%) patients were lost to follow up. 5, 2, and 1 patient died among Proven, Probable and Possible Aspergillosis cases respectively. The high mortality (83.3%) among this group suggests graveness of this disease in patients having all the risk factors and clinical and mycological evidences suggestive of IPA.

Thus, early diagnosis of secondary Aspergillosis in such patients may be of great importance, because early antifungal treatment is associated with an improved outcome.

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Volume 3 Issue 4 August 2016