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Original Article

MYCOLOGICAL AND SEROLOGICAL STUDY OF PULMONARY ASPERGILLOSIS IN CENTRAL INDIA

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Abstract

Purpose: To study the prevalence and predisposing factors of *Aspergillus* infection and correlate microscopic, culture and serological findings along with drug sensitivity. **Methods**: Sputum samples from 123 patients of pulmonary disease with clinical suspicion of having fungal, especially *Aspergillus* infections, were examined microscopically and for culture. Minimum inhibitory concentration (MIC) of itraconazole was tested against the isolates. Serum samples from these patients were tested for precipitin against *Aspergillus* antigen using immunodiffusion (ID) technique. **Results**: *Aspergillus species* were isolated in 20 (16.26%) cases and *Aspergillus fumigatus* was the predominant species isolated in 16 (80%) cases. Precipitins were detected in 29 (23.58%) cases. Serum samples collected from 50 healthy individuals to serve as controls showed no precipitin against *Aspergillus* antigen galactomannan. This fungus was found to be sensitive to itraconazole with MIC range 0.125-1 □g/mL. **Conclusions**: Serological tests have an edge over routine smear and culture methods for the diagnosis of pulmonary aspergillosis. Itraconazole is more effective than amphotericin B and fluconazole in the treatment of aspergillosis.

Key words: *Aspergillosis, prevalence, serodiagnosis, drug sensitivity*

Amongst the various mycoses of man, aspergillosis is one of the earliest fungal diseases to be recognized. *Aspergillus* spp. are ubiquitous fungi, commonly occurring in soil, water, and decaying vegetation. *Aspergillus* spp. have been cultured from unfiltered air, ventilation systems, contaminated dust dislodged during hospital renovation and construction, horizontal surfaces, food, and ornamental plants.[1] The most important nosocomial infection due to *Aspergillus* spp. is pneumonia,[2,3] the primary route of acquiring infection being inhalation of the fungal spores. From lungs, dissemination may take place leading to systemic aspergillosis. Occurrence of *Aspergillus* infection in association with chronic lung diseases like pulmonary tuberculosis, lung abscess and bronchopneumonia with residual lung cavity, asthma and lung malignancy is documented.[4] The diagnosis of *Aspergillus* infection presents considerable difficulty. The signs and symptoms in most cases of aspergillosis are non-specific, and radiological findings are of little diagnostic help. Diagnosis of aspergillosis requires the isolation and identification of the fungus. Galactomannan antigen-based serologic assays are now being developed in an attempt to allow for the rapid and specific diagnosis of *Aspergillus* infections; though their clinical usefulness is presently undefined.[5-7] In addition to diagnosis, the treatment of aspergillosis is also controversial.[8,9] The present study was undertaken to find correlation between microscopic findings, culture and serodiagnosis of *Aspergillus* infection. We also studied the prevalence of various species of *Aspergillus* and predisposing factors for *Aspergillus* infection and drug sensitivity.

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Materials and Methods

Subjects

The study was carried out at the department of Microbiology, Government Medical College and hospital, Nagpur. The procedure followed was in accordance with ethical standards on human beings. A total of 123 cases of chronic pulmonary infections: Pulmonary TB (57), lung abscess (15), lung malignancy (8), chronic bronchitis (13), pleural effusion (13) and bronchopneumonia (6) with the suspicion of secondary invasion by *Aspergillus* on the basis of clinical and radiological findings, were included in the present study. In addition to this, 11 cases with suspected allergic bronchopulmonary aspergillosis were also included.

Tests

At least two samples of expectorated morning sputum samples on two consecutive days were collected from each patient in a wide mouthed autoclaved sterile

glass bottle and examined for fungi by microscopic examination of 10% KOH mount and cultured on Sabouraud dextrose agar (SDA) medium. Isolated *Aspergillus* species were identified as per standard methods.[10] Susceptibility to amphotericin B, itraconazole and fluconazole was tested by determining minimum inhibitory concentration by agar dilution method at Ranbaxy Research Laboratory. Serum was collected from cases as well as age and sex matched healthy controls and tested for the presence of *Aspergillus* precipitin by agar gel double diffusion test, using antigen prepared from the liquid culture media.[11]

Preparation of antigen

Culture filtrate antigen of *A.fumigatus* and *A.niger* was prepared using two strains of each species. Steps employed for preparation of antigen in brief were:

Preparation of *Aspergillus* culture

grinding

Defatting

Extraction

Clarification

Sterilization

The *Aspergillus* was grown for 3-4 weeks in a synthetic medium glucose asparagine broth as stationary culture. The mycelial mat were separated and transferred to 95% acetone and kept for 24 hours. Then it was squashed gently between two layers of filter paper and dried in desiccator with anhydrous calcium chloride and then finely pulverized in pestle and mortar. Solvent ether was used for defatting. The active allergenic substances from the defatted powder were extracted with highly alkaline buffer (pH 8.0) and soluble active ingredients were separated by filtration through Whattman No.1 filter paper. Allergenic extracts were sterilized by filtration through millipore filter paper.

Agar gel diffusion test[12]

The test was done in 1.5% purified agar gel in barbitone buffer (pH 8.6) on microscope slides. After pouring the gel it was allowed to set for 10 minutes then it was transferred to the moist chamber and kept at 4ºC for the next 45 to 60 minutes. The wells were punched in the gel and charged with *Aspergillus* antigen solution and test samples (sera). The standard positive sera were regularly employed as control. The slide was transferred to moist chamber and kept at 4ºC for 48 hours for immunodiffusion and examined under oblique illumination for immunoprecipitate between the wells of test and antigen.

Results

In the present study, out of 123 cases, confirmed etiology of pulmonary aspergillosis was established in 20 (16.26%) cases. Ten cases were positive by either KOH preparation or culture and were considered of doubtful etiology. In all positive cases repeat culture was done to confirm etiology (Table 1). *A.fumigatus* was the commonest species isolated; followed by *A.niger* and *A.flavus* (Table 2).

Table 1 : Microscopy and culture positivity in sputum for *Aspergillus* in 123 cases of pulmonary infections

Microscopy (KOH mount)	Culture		Total
	Positive	Negative	
Positive	20	4	**24**
Negative	6	93	**99**
Total	**26**	**97**	**123**

Table 2 : Prevalence of different *Aspergillus* species in various bronchopulmonary Diseases

Pulmonary diseases (n)	*Aspergillus* species isolated			Total
	A. fumigatus	*A. niger*	*A. flavus*	
Pulmonary tuberculosis (57)	11	2	1	**14**
Asthma (11)	1	-	-	**1**
Malignancy (8)	3	-	-	**3**
Lung abscess (15)	1	1	-	**2**
Chronic bronchitis (13)	-	-	-	**-**
Pleural effusion (13)	-	-	-	**-**
Bronchopneumonia (6)	-	-	-	**-**
Total (123)	**16**	**3**	**1**	**20(16.26%)**

All culture positive cases of pulmonary aspergillosis were also positive by agar gel double diffusion test, Well-defined intense precipitates were observed in 23.58% serum samples. Twenty-eight sera reacted with *A.fumigatus* antigen while only 3 reacted with *A.niger* antigen. A serum sample from a case with *A.flavus* also reacted with *A.fumigatus* antigen in the present study. The correlation between serological results and culture is shown in (Table 3).

Table 3 : Correlation between serological findings and culture of *Aspergillus*

Agar gel diffusion	Culture		Total
	Positive	Negative	
Positive	26	3	**29**
Negative	0	94	**94**
Total	**26**	**97**	**123**

Antiaspergillus antibodies were not detected in any of the controls. All strains of *Aspergillus* isolated in the study were also tested for antifungal susceptibility to three drugs, amphotericin B, itraconazole and fluconazole (Table 4). Itraconazole was found to be more active (MIC range 0.5-2□g/mL).

Discussion

Comparison of the prevalence of *Aspergillus* in sputum shows that it occurs with greater frequency in malignancy and tuberculosis patients than other pulmonary diseases. Occurrence of *Aspergillus* infection in association with chronic lung disease is documented.[13]

Table 4: Antifungal susceptibility of *Aspergillus* spp.

Aspergillus spp (n)	Amphotericin B □g/mL		Itraconazole □g/mL		Fluconazole □g/mL		
	0.5-2	>2	0.125-1	>1	0-8	8-64	>64
A.fumigatus (16)	14	2	16	0	_	_	16
A.niger (3)	2	1	3	0	_	_	3
A.flavus (1)	1	0	1	0	_	_	1
Total (20)	**17**	**3**	**20**	**0**	_	_	**20**

Susceptibility to *Aspergillus* appears to be enhanced by certain factors like therapy with antibiotics and chemotherapeutic drugs and steroids, depression of bone marrow activity, carcinoma and leukaemia. Especially antibiotics and steroids appear to be more significant as they may stimulate the growth and virulence of infecting fungus by destruction of competing bacterial flora. Common use of broad spectrum antibiotics in the pulmonary disease for long duration, for example in pulmonary tuberculosis, lung abscess, and chronic bronchitis may predispose to fungal infection.

The isolation of *Aspergillus* from sputum is often considered a contaminant. Whether the isolated agent is a contaminant or indeed responsible for the pathological condition is often questionable. Routine blood cultures are not much help as they are remarkably insensitive[14] and systemic antibody responses in immunocompromised patients are likely to be unreliable indicators of infection.[15, 16] However, seropositivity detected in the present study correlates with results of similar studies[17-19] and suggests that serological tests have an edge over routine culture for diagnosis of pulmonary aspergillosis. Detection of antibodies indicates that there is definite infection induced antibody production and thus rules out the presence of fungus as mere contaminant. Pulmonary aspergillosis occurs in immunocompromised hosts in whom immune response is less or may be absent.[20] In such cases serological findings may be absent. Therefore, such cases with negative serological findings may not be considered negative for infection. Thus a combination of culture and serology can be ideal to confirm the diagnosis of pulmonary aspergillosis.

There have been no convincing demonstrations that treatment failure in patients with aspergillosis can be attributed to the development of amphotericin B resistance. In the present study the results of invitro susceptibility testing of 20 isolates of *Aspergillus* species show that itraconazole was more active than amphotericin B (MIC range 0.5-2 □g/mL) and fluconazole (MIC 0-64□g/mL).

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