

Secondary Aspergillus in Bronchoalveolar Lavages (BALs) of Pulmonary Tuberculosis Patients from North-India

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Abstract: To find out the prevalence of *Aspergillus* spp. in Bronchoalveolar Lavages (BALs) of pulmonary tuberculosis patients, to study the anti-*Aspergillus* antibodies in patient's sera and to study the anti-fungal susceptibility of the isolated *Aspergillus* strains. BALs obtained from sixty-five patients of pulmonary tuberculosis and 10 healthy volunteers were studied. Direct microscopy was performed by 10% KOH and Lacto-Phenol Cotton Blue (LPCB) mounts and fungal cultures were performed on two sets of Czapek Dox agar (CDA) after adding chloramphenicol (0.05 mg/ml). Minimum Inhibitory Concentrations (MICs) were determined for fluconazole, itraconazole and amphotericin B by broth micro-dilution method. Finally, fungal serology was performed by Double Immunodiffusion (DID), Enzyme Linked Immunosorbent Assay (ELISA) and Dot Blot Assay (DBA) to evaluate the efficacies of these tests for detection of anti-aspergillus antibodies. *Aspergillus* species were isolated from 13.8% (9/65) patients. *A. fumigatus* was isolated in 5 (55.5%) patients while *A. flavus* and *A. niger* were isolated from 3 (33.3%) and 1 (11.1%) patients respectively. None from the control group showed growth of any *Aspergillus* species. All the *Aspergillus* species were consistently resistant to fluconazole (MIC >16 µg/ml). MICs for itraconazole and amphotericin B ranged between 0.12- >16 µg/ml and 0.12- 0.5 µg/ml, respectively. Anti-aspergillus antibodies were detected in 14 (21.5%) patients by DID as well as by ELISA; whereas, 12 (18.4%) patients showed presence of anti-aspergillus antibodies by DBA. The prevalence of *Aspergillus* in BAL of pulmonary tuberculosis patients was 13.8%. *Aspergillus fumigatus* was the predominant species, however the isolation of *A. flavus* (33.3%) was also significant. We suggest that the diagnostic tests for Aspergillosis, including culture and serology, should be incorporated in routine clinical laboratories so that we may not miss the secondary Aspergillosis in tuberculosis patients.

Key words: Secondary aspergillosis • tuberculosis • *Aspergillus* spp. • fungal serology • North India

INTRODUCTION

There has been an increasing recognition of respiratory mycoses, including Aspergillosis, in this country in the recent past [1]. Similarly, tuberculosis in India, as in other developing countries, is a major public health problem [2]. Nearly 40% of the Indian population is infected with *Mycobacterium tuberculosis* and 1.5-2.0% of this population is suffering from radiological active disease [2]. Since a large proportion of population is suffering from tuberculosis and as the pre-existing lung diseases, including tuberculosis, are being documented to cause secondary Aspergillosis, we planned the present study to find out the prevalence of *Aspergillus* spp. in Bronchoalveolar Lavages (BALs) of pulmonary

tuberculosis patients; to study the anti-*Aspergillus* antibodies in patient's sera; and to study the anti-fungal susceptibility of the isolated *Aspergillus* strains.

MATERIALS AND METHODS

The present study was conducted on patients of pulmonary tuberculosis, on whom the bronchoscopy was performed for some reasons, attending the outpatient or admitted in inpatient department of TB and Chest Diseases, J.N. Medical College and Hospital, A.M.U. Aligarh during the period of 2 years. The criteria [3] used for the diagnosis of pulmonary tuberculosis was (i) if the clinical features were suggestive of pulmonary tuberculosis (ii) sputum smear revealed acid fast bacilli

and/or sputum culture grew *Mycobacterium tuberculosis* (iii) radiological features on chest radiograph were compatible with tuberculosis.

Sixty-five patients were selected for the study. Ten, age and sex matched healthy volunteers were also included after taking the informed consent. None of the selected patients had received any antifungal therapy prior to the study.

Bronchoscopy was performed according to Wimberley *et al.* [4]. Three consecutive BAL specimens were collected in sterile vials. Blood was collected in EDTA and plain vials for routine hematological investigations and fungal serology respectively. Sera were separated and stored at -20°C till further testing.

Homogenized BAL specimens were subjected to direct microscopy by making 10% KOH mount and Lacto-Phenol Cotton blue (LPCB) mount. BALs were streaked on two sets of Czapek Dox Agar (CDA) [5] after adding chloramphenicol (0.05 mg/ml) and were incubated at 25 and 37°C. The *Aspergillus* species isolated in the culture were identified as per standard methods [5, 6].

Antifungal susceptibility testing: Antifungal susceptibility to fluconazole, itraconazole and amphotericin B was tested, and Minimum Inhibitory Concentrations (MICs) were determined for each fungal isolate by using broth micro-dilution method [7].

Fungal serology: Antigen preparation: Antigens of *A. fumigatus*, *A. flavus* and *A. niger* were prepared separately as described by us earlier [8, 9].

Double Immunodiffusion (DID): DID was carried according to Ouchterlony's technique [10] as adopted previously [8,9].

Enzyme linked immunosorbent assay (ELISA): ELISA was performed according to the method of Kauffman *et al.* [11] with some modifications as described earlier [9].

Dot Blot assay (DBA): DBA was performed according to the method described by us elsewhere [12].

RESULTS

The mean age of the patients was 32±3 years and of which, 66.2% were males and 33.8% were females. The presenting features of the patients are presented in Fig. 1.

Aspergillus isolation in culture/direct microscopy: *Aspergillus* species were repeatedly isolated from BAL

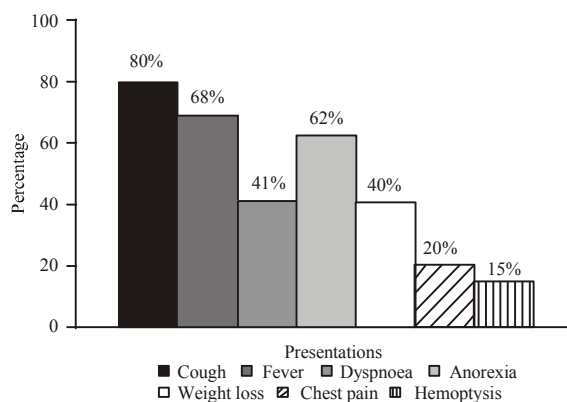


Fig. 1: Presenting features of the patients included in the study

Table 1: MICs (µg/ml) of the antifungal agents tested against different *Aspergillus* species

<i>Aspergillus</i> species	Fluconazole	Itraconazole	Amphotericin B
<i>A. fumigatus</i>	>16	>16	0.12
<i>A. fumigatus</i>	>16	>16	0.25
<i>A. fumigatus</i>	>16	0.12	0.12
<i>A. fumigatus</i>	>16	0.12	0.12
<i>A. fumigatus</i>	>16	0.12	0.12
<i>A. flavus</i>	>16	0.12	0.25
<i>A. flavus</i>	>16	0.12	0.25
<i>A. flavus</i>	>16	0.25	0.50
<i>A. niger</i>	>16	0.12	0.25
<i>A. fumigatus</i> 1008*	>16	0.06	0.06
<i>A. fumigatus</i> SI-I*	>16	0.12	0.06

**A. fumigatus* 1008 and *A. fumigatus* SI-I were used as control strains

culture of 9 (13.8%) patients. Of these culture positive cases, microscopic examination revealed fungal elements in 77.7% (7/9) cases. None of the culture negative case showed presence of fungal elements in direct microscopy.

Frequency of *Aspergillus* species isolation: *A. fumigatus* was isolated in 5 (55.5%) patients while *A. flavus* and *A. niger* were isolated from 3 (33.3%) and 1 (11.1%) patients respectively. None from the control group showed growth of any *Aspergillus* species.

Antifungal susceptibility: The results of the antifungal susceptibilities indicating the MIC values to itraconazole, fluconazole and amphotericin B are shown in Table 1.

Serodiagnosis: Among the serological tests, anti-*Aspergillus* antibodies were detected in 14 (21.5%) patients by DID as well as by ELISA. However, 12 (18.4%) patients showed presence of anti-*Aspergillus* antibodies

by DBA. It was observed that the cases detected by DID and DBA were also positive by ELISA. Cross-reaction between antigens of *A. fumigatus* and *A. flavus* was noticed in this study. However, no cross-reaction with *A. niger* antigen was noticed.

DISCUSSION

Bronchopulmonary Aspergillosis is fairly common in India and could be seen as an important emerging disease however; its diagnosis is usually missed due to lack of pathognomonic clinical features [13] and paucity of diagnostic mycological laboratories [8, 9]. The Indian data regarding the prevalence of *Aspergillus* in BAL of respiratory patients is elementary; therefore the need to perform more studies in this aspect is mandatory. In the present study, the prevalence of *Aspergillus* in BAL of pulmonary tuberculosis patients was 13.8%. Our findings related to the prevalence is in accordance with previous published reports [5, 13] however, it is much lower than the prevalence reported by Kurhade *et al.* [14]. *Aspergillus fumigatus* was the predominant species isolated from BAL in the present study and correlates well with results of similar other studies [14, 15]. The frequency of isolation of *A. flavus* (33.3%) in the present study is higher than other studies [5, 14, 15] but is in accordance with our previously published report [16]. The variations in the prevalence and frequency of isolation of various *Aspergillus* species may be due to the geographical variations.

In the present study the seroprevalence of Aspergillosis in pulmonary tuberculosis was found to 21.5% similar to the findings of Kurhade *et al.* [14]. It was noticed that anti-*Aspergillus* antibodies were detected equally by DID and ELISA and all the cases positive by DID were also positive by ELISA. However, anti-*Aspergillus* antibodies were detected in 18.4% cases by DBA, which may be due to subjective error in interpreting the results of DBA. Analyzing the serodiagnostic tests, it is being suggested that the laboratories that do not have the facilities for performing ELISA may opt for DID as a cheaper and easier substitute to diagnose secondary Aspergillosis in patients of pulmonary tuberculosis. DBA, which is again easier to perform and do not require sophisticated equipments, could be used as a confirmative test however, the sensitivity of this test was found to be lower than DID and ELISA in this study. We therefore suggest that further studies are required to

evaluate the usefulness of DBA in diagnosis of Aspergillosis in pulmonary tuberculosis patients. It is also suggested that as the pulmonary Aspergillosis mimics pulmonary tuberculosis, patients not responding to anti-tubercular therapy should be searched for Aspergillosis by performing culture as well as serological tests. It would be wise if we include, as a routine, the diagnostic tests for Aspergillosis so that we may not miss the secondary Aspergillosis in tuberculosis patients.

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