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Colonization of Aspergillus Species in COPD Patients and Their Antifungal Suceptibility

Priyanka Choudhary*, Pooja Singh Gangania*

Author Affiliation
*Research Scholar, Department of Microbiology, Santosh Medical College and Hospital, Ghaziabad, U.P.

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Priyanka Choudhary, Research Scholar, Department of Microbiology, Santosh Medical College and Hospital, Ghaziabad, Uttar Pradesh 201009.
E-mail: priyankateotia2013@gmail.com

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Abstract

Objectives: To isolate *Aspergillus* species from lung infection in COPD patients and to determine Antifungal Susceptibilty against Amphotercin B by E-Test Strip method. *Materials and Method:* Sputum sample collected from total 54 patients visiting TB and Chest department of Santosh Hospital. All are above 18 year of Age and suffering from COPD. *Aspergillus* species were isolated by culturing samples on SDA which were confirmed by conventional method. Antifungal Susceptibilties were determined by E-Test strip method on Muller Hinton Agar with Methylene Blue Dye . E-Test minimum inhibitory concentrations (MIC) of Amphotericin B determined. *Result:* 33% of *Aspergillus* species were isolated from sputum samples of COPD patients. *Conclusion:* The analysis of the present study concludes that *Aspergillus* is one of the major cause of colonization in COPD patients,especially males within age group(41-60) years, chronic smoking increases the rate of *Aspergillus* colonization and Amphotericin B gives a poor result in treatment.

Keywords: Chronic Obstructive Pulmonary Disease; Aspergillus Fumigatus ; Aspergillus Flavus ; Aspergillus Niger; Aspergillus Terreus; Lactophenol Cotton Blue; Sabourad's Dextrose Agar; Potato Dextrose Agar; Minimum Inhibitory Concentration; Elipsometer Test.

Introduction

Members of the genus *Aspergillus* are ubiquitous moulds widely distributed in the environment. About 185 different species of *Aspergillus* have been identified, out of which 20 are declared pathogenic.

Aspergillus spores, upon inhalation, can lead to colonization, allergic manifestations or invasive infection depending on host immunity. Invasive aspergillosis is the second most common invasive fungal infections in humans [1].

COPD is a common , preventable lung disorder characterized by progressive, poorly reversible air flow limitation often with systemic manifestation, in response to tobacco smoke and other harmful inhalation exposures. Patients with severe copd who often receive broad-spectrum antibiotics and corticosteroids are becoming one of the main risk groups for Invasive pulmonary aspergillosis [2].

Majority (80%) of invasive infections caused by *A. fumigatus* and the second most frequent (15-20%) pathogenic is *A. flavus* and to a lesser extent *A. niger* and *A. terreus* but now *A.* flavus is overcoming

A.fumigatus. *A. flavus*, with its unique ability to survive at higher temperatures is making it, most predominant pathogen in arid dry weather countries like India [1].

Materials and Methods

Expectorated morning sputum samples were collected from each patient in a wide mouth sterile disposable plastic container, total 60 patients sputum sample were taken, who belongs to above 18 year of age having history of cough with sputum production, shortness of breath, wheezing sound, smoking from long time and those who have conformed COPD history.

All sputum samples were cultured on SDA (Sabourad's dextrose agar) with Chlorhexidine and incubated at 32-37^{0}c for 3 to 4 days or a week for isolation of *Aspergillus* species. Identification and confirmation is done on the basis of Conventional method such as colony characterstics, LPCB (Lactophenol cotton blue) preparation and Slide culture.

After seven days, filamentous colonies were examined and *Aspergillus* spp. Identified based on macroscopic and microscopic methods. Species are differentiated by morphological characterstics and colour. Macroscopically, colonies are flat, granular, downy to powdery in texture often with radial grooves. Colony surface is yellow initially but turns dark yellowish green with age on SDA agar.

Microscopically,hyphae are septate and hyaline branching at 45^0 angle. The conidiophores originating from supporting hyphae and terminate in vesicles at the apex [3].

Antifungal Susceptibility Testing

Preparation of Inoculum

All isolates were freshly sub–cultured on potato dextrose agar (PDA) slants to obtain good sporulation. The culture tubes were flooded with 1ml of 0.9% saline and vortexed for 15 seconds to dislodge the conidia. The growth suspensions were transferred to another sterile tube containing 1.5 ml saline and 0.2% Tween 80. A conidial suspension containing approximately 1x10^6-5x10^6 cells was used as inoculum [4].

E-TEST

(MHA) Mueller- hinton agar with 2% glucose and methylene blue (0.5 ug/ml) (MH-GMB) were used for Etest. Etest was performed according to manufacturer's instructions. Briefly, each 150-mm petri plate containing 60 ml of medium was inoculated by streaking the swab over the entire surface of the medium. Before apply the E strips, the plates were allowed to dry for 15 min. Etest antifungal susceptibility strips for Amphotericin B were stick on the plate. MIC reading were taken after 24 hour of incubation at 35^0 C. The Etest MIC was define as the lowest drug concentration at which the border of the elliptical inhibition zone intercepted the scale on the antifungal strip, microcolonies within the zone were ignored [4].

Result

Distribution of Aspergillus Species

Total no of *Aspergillus* species	*Aspergillus fumigatus*	*Aspergillus flavus*	*Aspergillus niger*	other isolates
18(25%)	12(23%)	3(6%)	3(6%)	5(9%)

A total of 54 possible patients with severe exacerbation of COPD were evaluated. Of these most of belong to age group 41-60(64%) years. Isolation of *Aspergillus* species was higher in males compared to females due to their addictive habit of smoking. Total 18(25%) *Aspergillus* species isolated, 12(23%) were identified as *A.fumigatus*, 3 (6%) as *A.flavus* and 3(6%) were *A.niger*. Antifungal susceptibility by Etest method showed only 1 strain to be sensitive for Amphotericin B had MIC range 2-3mcg/ml while other 17 were resistant.

Sensitivity Pattern

Antibiotic Pattern	No of Patients
Senstive	1
Resistant	17

Discussion

One of the first question that arises when physicians face cultures positive to *Aspergillus* from lower respiratory samples in non immunocompromised: Is there colonisation or infection?, should the patient be treated with antifungals?, and what should be is the prognosis?, that is, how to interpret and manage patients from which *Aspergillus* is obtained. The answer to the question is important since an early diagnosis is crucial to improve prognosis. It has been postulated that isolation of an *Aspergillus* species from respiratory samples in critically ill patients (even when immunocompetent) should not be routinely discarded as colonisation, but in elderly patients (commonly having underlying diseases) isolation is usually interpreted as colonization [5].

In our study a total of 54 COPD patients were included of which 43(80%) were males and 11(20%) were females. The prevalence of males having COPD was higher than females due to their frequent smoking habits, field work, labour work and higher outside exposure etc. while females were only infected by bio mass fuel cooking, as less smoking habits are found in females compared to males [2].

In the present study we have observed that most of the COPD patients belonged to age group between 41-60 years due to their continuing smoking habits.

Of the 54 COPD patient a total number of 18(33%) *Aspergillus* species were isolated. Of these 17(94%) were isolated from males while only one(6%) was isolated isolated from a female patient. The result of our study was similar to a study by AM khurhade et.al, in which 16.26% of *Aspergillus* species were isolated from COPD patients [6]. Recently a large, retrosepective study conducted by Guinea et.al analyzed the incidence of *Aspergillus* species isolation from lower respiratory tract samples in patients admitted for AECOPD in tertiary hospital, the authors found 22% *Aspergillus* isolation [7].

In our study we isolated most of the *Aspergillus* species from the males, which was 39%. This was in contrast to a study by Mahesh et. al, in which they found 11.1% males to be were infected by *Aspergillus* species. This is due to the fact that males are highly involved in addictive habits like smoking, alcoholism etc and some are infected due to their occupations like diary farms, farmers, labour etc. as a result they are highly exposed to dust, smoke, hazardious chemicals etc which lead to respiratory infections [8].

A study by Arturo Huerta et. al, reported that *A.fumigatus* was isolated in 25(17%) cases out of 144, *A.niger* in 1(0.69%) and *A.flavus* in 1(0.69%) [7]. A study by Kurhade et. al is also showed similar result in which *Aspergillus fumigatus* is isolated in 16(13%) cases out of 123 cases, *A.niger* in 3(2.4%) and *A.flavus* in 1(0.81%) [47]. Another study by Barberan et. al showed 16(15%) cases positive for *A.fumigatus* of the 106 samples, 1(0.94) isolated positive for *A.niger* and 1(0.94) positive for *A.flavus [9]*. In the present study also of the 18 *Aspergillus* species isolated, 12(66%) were identified as *A.fumigatus*, 3(16%) as *A.flavus*, 3(16%) as *A.niger* and 6(33%) were other isolates like Candida species, Rhizopus and Penicillum.

The prevalence of *Aspergillus* spp. isolation may have been higher if we had used bronchoscopic techniques and specific culture media. However, in real-life settings, clinicians often only have access to sputum samples. In a recent study, Phasley et al [10] reported that the isolation of *A.fumigatus* in sputum culture was significantly higher using a research approach compared to the standard method for mycological investigations. Previous studies, which have not focused solely on *Aspergillus spp.*, have found different prevalence rates of fungi isolation in respiratory samples from patients with cystic fibrosis, COPD and asthma. Recently, a large, retrospective study conducted by Guinea et al [11], analyzed the incidence of *A.fumigatus* isolation from lower respiratory tract samples in patients admitted for AECOPD in a tertiary hospital. The authors found 239 isolations of *Aspergillus* species (16.3 per 1000 admissions), but only 53 (22%) patients had probable IPA. However, unlike our prospective study, the fungal isolations were detected retrospectively by the microbiology laboratory. There is no doubt that COPD patients are a population at risk for *Aspergillus spp.* colonization. In a previous study of critically ill patients, *Aspergillus* spp. isolation from respiratory secretions was significantly associated with both an underlying diagnosis of COPD and treatment with corticosteroids [12]. These findings have been confirmed by other authors, and have strengthened the relationship between pulmonary infection with *Aspergillus spp.* and the use of intravenous corticosteroids in COPD patients admitted to the ICU for severe exacerbation. In contrast, a study conducted by Afessa et al [13] reported no isolation of *Aspergillus spp.* in the respiratory specimens from 250 COPD patients admitted to the ICU because of acute respiratory failure, although no report on corticosteroid therapy was performed.

Antifungal susceptibility testing has become an important tool for physician faced with making

difficult treatment decisions regarding treatment of patients with fungal infections.

In present study we determined antifungal susceptibility of isolated *Aspergillus* strains of which only one strain was found to be sensitive for Amphotericin B with MIC range 2-3mcg/ml and the remaining strains were found to be resistant for Amphotericin B with MIC range >32mcg/ml. The result of our study are similar to a study by Khurhade et al who showed that only 2 *Aspergillus* strains were found sensitive for Amphotericin B (MIC range 0.5-2ug/ml) of the 20 *Aspergillus* strains isolated. A study by Barberan et al however showed all fungal isolates from 65 patients to be resistant against Amphotericin B.

The results of our study were in contrast to a study by Al wathiqui et.al in which from the total 92 patients, 69 isolates were inhibited by Amphotericin B (0.064-4 to 3ug/ml).

Susceptibility testing are carried out by a broth microdilution test and disc diffusion. MICs are determined after 48 h by the reference broth microdilution method, and after 24 and 48 hours by disc diffusion. As others studies have shown, the broth microdilution and disc diffusion produced comparable MICs and a good level of agreement for all *Aspergillus spp*.

The medium employed for the disc diffusion method was Mueller–Hinton agar (Difco) supplemented with 2% glucose and Methylene Blue (0.5 mg/L). This medium is recommended in the document M44-P for disc diffusion susceptibility testing for yeast because of its enhanced growth and simplified reading relative to the broth microdilution method. 31 Zone size measurements are subjective, and this adds an important source of variability to the test; however, our isolates showed zone diameters with very clear border edges in the Mueller-Hinton agar.

Our finding may serve to purpose future more comprehensive studies, with biological basis that includes pulmonary and systemic markers of the immune and inflammatory response, in order to determine the role of this fungus in COPD exacerbations. On basis of our results, it appears that the E- test method is a useful method for testing the activity of drugs against *Aspergillus species* and Amphotericin B were found to be highly resistant against the *Aspergillus family*.

Conclusion

The analysis of the present study concludes that *Aspergillus* is one of the major cause of colonization in COPD patients,especially males within age group (41-60) years, chronic smoking increases the rate of *Aspergillus* colonization and Amphotericin B gives a poor result in treatment.

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