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Colonization of Aspergillus Species in COPD Patients and Their Antifungal Suceptibility

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ABSTRACT:
Objectives:ToisolateAspergillusspeciesfromlunginfectioninCOPDpatientsandtodetermineAntifungalSusceptibiltyagainstAmphotericinBbyE-TestStripmethod.MaterialsandMethod:Sputumsample 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 Susceptibiltieswere determined by E-Test strip method on Muller Hinton Agar with MethyleneBlueDye.E-Testminimuminhibitoryconcentrations(MIC) ofAmphotericinBdetermined.Result:33%ofAspergillusspecieswere isolated from sputum samples of COPD patients. Conclusion: The analysis of the present study concludes that Aspergillus is one of the majorcauseofcolonizationinCOPDpatients,especiallymaleswithinagegroup(41-60)years,chronicsmokingincreasestherateofAspergilluscolonizationandAmphotericinBgivesapoorresultintreatment.

Keywords: Chronic Obstructive Pulmonary Disease; Aspergillus Fumigatus; Aspergillus Flavus; Aspergillus Niger; Aspergillus Terreus; LactophenolCottonBlue;Sabourad'sDextroseAgar; PotatoDextrose Agar; Minimum Inhibitory Concentration;ElipsometerTest.

I. INTRODUCTION

Members of the genus Aspergillus are ubiquitous moulds widely distributed in the environment. About 185 different species of Aspergillus have been identified,outofwhich20aredeclaredpathogenic.
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 flowlimitation of ten with system icmanifestation,in response to tobacco smoke and other harmful inhalationexposures. Patientswithseverecopd who often receivebroad-spectrum antibiotics and corticosteroids are becoming one of the main risk groupsfor Invasivepulmonaryaspergillosis[2].
Majority(80%)ofinvasiveinfectionscausedbyA. fumigatusand the second most frequent (15-20%) pathogenicisA.flavusandtoalesserextentA.nigerand A. terreusbut now A. flavus isovercoming A.fumigatus.A.flavus, withitsuniqueabilitytosurvive at higher temperatures is making it, most predominantpathogeninariddryweathercountries like India[1].

II. MATERIALS AND METHODS

Expectorated morning sputum samples were collected from each patient in a wide mouth sterile disposableplasticcontainer,total60patientssputum sampleweretaken,whobelongstoabove18yearof age having history of cough with sputum production, shortnessofbreath,wheezingsound,smokingfrom 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(fig. 1) Identification and confirmation is done on the basis of Conventional method such as colony characterstics, LPCB (Lactophenolcotton blue) preparation(fig. 2)and Slide culture.

After seven days, filamentous colonies were examined and Aspergillus spp. Identified based on macroscopic and microscopic methods.

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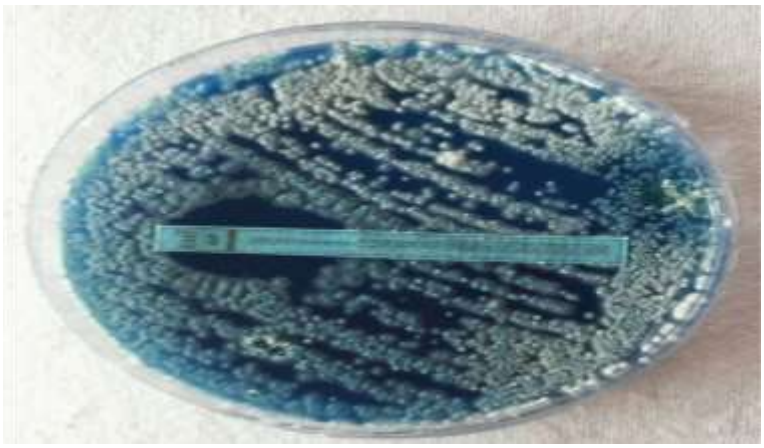
Species are differentiated by morphological characterstics and colour. Macroscopically, colonies are flat, granular, downy to powdery in texture often with radial grooves. Colony surface is yellow initially but turns dark yellowish green with age on SDA agar.
Microscopically,hyphae are septate and hyaline branchingat45^{0}angle.Theconidiophoresoriginating fromsupportinghyphaeandterminateinvesiclesat 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 of0.9%saline andvortexed for15secondstodislodge the conidia. The growth suspensions were transferredtoanothersteriletubecontaining1.5ml 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-hintonagarwith2%glucoseand methyleneblue(0.5ug/ml)(MH-GMB)wereusedfor Etest. Etestwas performedaccording to manufacturer's instructions. Briefly, each 150-mm petri plate containing 60 ml of medium was inoculated by streaking the swab over the entire surfaceofthemedium.BeforeapplytheEstrips,the plates were allowed to dry for 15min. Etestantifungal susceptibility strips for Amphotericin B were stick ontheplate.MICreadingweretakenafter24hourof incubationat35^{0}C.(fig. 3)TheEtestMICwasdefineasthe lowestdrugconcentrationatwhichtheborderoftheellip ticalinhibitionzoneinterceptedthescaleontheantifung alstrip,microcolonieswithinthezonewere ignored[4].

(fig.1) Aspergillus fumigatus on SDA Agar
(fig.2)LPCB staining of Aspergillus



(fig. 3) Aspergillus flavusShowing Zone Of Sensitivity Against Amphotericin B

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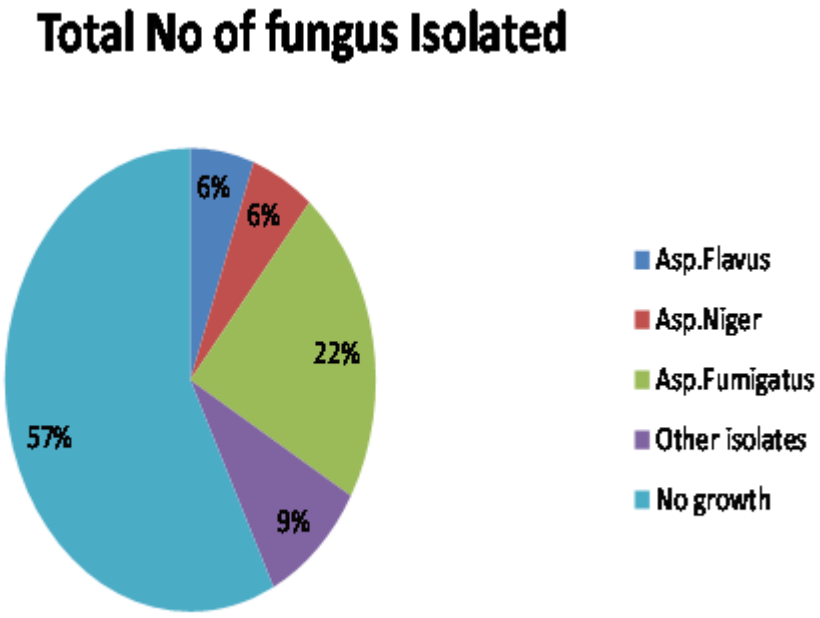
III. RESULT

Distribution of Aspergillus Species

Total no of Aspergillusspecies other isolates	Aspergillusfumigatus	Aspergillusflavus	Aspergillusniger
18(25%) 12(23%)	3(6%)	3(6%)	5(9%)

A total of 54 possible patients with severe exacerbationofCOPDwereevaluated.Ofthesemost ofbelongtoagegroup4160(64%)years.IsolationofAs pergillusspecieswashigherinmalescomparedtofemal esduetotheiraddictivehabitofsmoking.Total 18(25%) Aspergillus species isolated, 12(23%)were identifiedasA.fumigatus,3(6%)asA.flavusand3(6%) were A.niger (fig. 4). Antifungal susceptibility by Etestmethod showed only 1 strain to be sensitive for Amphotericin B had MIC range 2-3mcg/ml while other 17 wereresistant.



(Fig. 4) Distribution Of Aspergillus Species

Sensitivity Pattern

AntibioticPattern	No of Patients
Senstive	1
Resistant	17

IV. DISCUSSION

One of the first question that arises when physicians face cultures positive to Aspergillusfrom lower respiratory samples in non immunocompromised: Is there colonisationor 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 crucialtoimproveprognosis.Ithasbeenpostulated thatisolationofanAspergillusspeciesfromrespiratory samples in critically ill patients (even when immunocompetent)shouldnotberoutinelydiscarded 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 includedofwhich43(80%)weremalesand11(20%) werefemales.TheprevalenceofmaleshavingCOPD washigherthanfemalesduetotheirfrequentsmoking

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habits, field work, labour work and higher outside exposureetc.whilefemaleswereonlyinfectedbybio massfuelcooking,aslesssmokinghabitsarefound infemalescomparedtomales[2].

Inthepresentstudywehaveobservedthatmostof the COPD patients belonged to age group between 41-60yearsduetotheircontinuingsmokinghabits.

Ofthe54COPDpatientatotalnumberof18(33 %) Aspergillus species were isolated. Of these 17(94%) were isolated from males while only one(6%) was isolatedisolatedfromafemalepatient.Theresultof 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, retrosepectivestudy conducted by Guinea et.al analyzedtheincidence ofAspergillusspeciesisolation from lower respiratory tract samples in patients admittedforAECOPDintertiaryhospital,theauthors found 22% Aspergillus isolation[7].

In our study we isolated most of the Aspergillus speciesfromthemales,whichwas39%.Thiswasin contrast to a study by Mahesh et. al, in which they found11.1%malestobewereinfectedbyAspergillus species. This is due to the fact that males arehighly involved in addictive habits like smoking, alcoholism etc and some are infected due to their occupations likediaryfarms,farmers,labouretc.asaresultthey are highly exposed to dust, smoke, hazardiouschemicalsetcwhichleadtorespiratoryinfe ctions[8].

A study by Arturo Huerta et. al, reportedthat A.fumigatuswasisolatedin25(17%)casesoutof144, A.nigerin 1(0.69%) and A.flavusin 1(0.69%) [7]. A studybyKurhadeet.alisalsoshowedsimilarresult inwhichAspergillusfumigatusisisolatedin16(13%) casesoutof123cases,A.nigerin3(2.4%)andA.flavusi n 1(0.81%) [47]. Another study by Barberan et. al showed 16(15%) cases positive for A.fumigatusof the106samples,1(0.94)isolatedpositiveforA.nigeran d 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.nigerand 6(33%) were other isolates like Candida species, Rhizopusand Penicillum.

The prevalence of Aspergillus spp. isolation may have been higher if we had used bronchoscopic techniques and specific culture media. However, in real-lifesettings,cliniciansoftenonlyhaveaccessto sputumsamples.Inarecentstudy,Phasleyetal[10] reportedthattheisolationofA.fumigatusinsputum culture was significantly higher using a research approach compared to the standard method for mycological investigations. Previous studies, which havenotfocusedsolelyonAspergillusspp.,havefound different prevalence rates of fungi isolation in respiratorysamplesfrompatientswithcysticfibrosis, COPD and asthma. Recently, a large, retrospective study conducted by Guinea et al [11], analyzed the incidence of A.fumigatusisolation 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),butonly53(22%)patientshadprobable IPA. However, unlike our prospective study, the fungal isolations were detected retrospectively by the microbiologylaboratory.ThereisnodoubtthatCOPD patients are a population at risk for Aspergillus spp. colonization. In a previous study of critically ill patients, Aspergillus spp. isolation from respiratory secretionswassignificantlyassociatedwithbothan underlying diagnosis of COPD and treatment with corticosteroids [12]. These findings have been confirmedbyotherauthors,andhavestrengthened the relationship between pulmonary infection with Aspergillus spp. and the use of intravenous corticosteroidsinCOPDpatientsadmittedtotheICU forsevereexacerbation.Incontrast,astudyconducted byAfessaetal[13]reportednoisolationofAspergillus spp. in the respiratory specimens from 250 COPD patients admitted to the ICU because of acute respiratory failure, although noreport on corticosteroid therapywas performed.

Antifungal susceptibility testing has become an important tool for physician faced with making difficulttreatmentdecisionsregardingtreatmentof patients with fungalinfections.

In present study we determined antifungal

susceptibilityofisolatedAspergillusstrainsofwhich only one strain was found to be sensitive for AmphotericinBwithMICrange2-3mcg/mlandthe 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 (MICrange0.5-2ug/ml)ofthe20Aspergillusstrains isolated.Astudy by Barberanetal however showed all fungal isolates from 65 patients to be resistant against AmphotericinB.

Theresultsofourstudywereincontrasttoastudy byAlwathiquiet.alinwhichfromthetotal92patients, 69 isolates were in hibited by AmphotericinB(0.064- 4 to3ug/ml).

Susceptibility testing are carried out by a broth

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microdilution test and disc diffusion. MICs are determined after 48 h by the reference broth microdilutionmethod,andafter24and48hoursby disc diffusion. As others studies have shown, the broth microdilution and disc diffusion produced comparableMICsandagoodlevelofagreementfor all Aspergillusspp.
Themediumemployedforthediscdiffusionmethod wasMueller–Hintonagar(Difco)supplementedwith 2% glucose and Methylene Blue (0.5 mg/L). This mediumisrecommendedinthedocumentM44-Pfor discdiffusionsusceptibilitytestingforyeastbecause ofitsenhancedgrowthandsimplifiedreadingrelative to the broth microdilution method. 31 Zone size measurements are subjective, and this adds an importantsourceofvariabilitytothetest;however, our isolates showed zone diameters with very clear borderedgesintheMueller-Hintonagar.
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.Onbasisofourresults,itappearsthat theE-testmethodisausefulmethodfortestingthe activity of drugs against Aspergillus species and Amphotericin B were found to be highly resistant against the Aspergillusfamily.

V. CONCLUSION

The analysis of the present study concludes thatAspergillus is one of the major cause ofcolonization inCOPDpatients,especiallymaleswithinagegroup(41 60)years,chronicsmokingincreasestherateofAspergi lluscolonizationandAmphotericinBgivesa poorresultintreatment.

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