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Molecular Detection Of*Aspergillus*Genus In Patients With Lower Respiratory Tract Infections From A Tertiary Care Centre In Central India.

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Abstract
Aspergillus spp. is frequently isolated in respiratory samples from patients with severe chronic obstructive pulmonary disease; however, the clinical significance of this mold is unclear and its presence may indicate temporary passage, benign chronic carriage, or onset of invasive disease. This hospital based cross-sectional observational study was carried out at the Department of TB and Chest, Index Medical College, Indore (M.P.). Detection of Aspergillus species was done using culture and Polymerase Chain Reaction. Sensitivity, specificity, of 10.0% KOH, and culture findings against PCR was found to be 68.7%, 95.1% respectively for KOH and 72.9% and 100.0% respectively for culture test. Species detection in nested PCR was A. niger (2.0%), A.fumigatus (11.3%) and A.flavus (10.0%). This shows more species specificity of PCR. Aspergillusfumigatus was the most dominant molds isolated from sputum in aspergillosis patients. Molecular methods are more sensitive and specific methods. It gives results for detection and speciation in short time which helps early diagnosis and treatment of patient.

Keywords: Aspergillus genus, Aspergillusfumigatus, lower respiratory tract infections (LRTI), polymerase chain reaction (PCR)

1. INTRODUCTION

Aspergillus is a diverse genus with a high economic, social and health impact, in which about 40 *Aspergillus* spp. are clinicallyrelevant. The *A. fumigatus*complexisthe major cause of aspergillosis worldwide, followed by the *A. Flavus*complex,the*A. niger* complex, and *A. terreus*.[1] *Aspergillus* spp. can cause a broad spectrum of pulmonary aspergillosis (PA), ranging from an allergic reaction (allergic bronchopulmonaryaspergillosis; ABPA) to various infections such as chronic pulmonaryaspergillosis (CPA) or invasive pulmonary aspergillosis (IPA).*Aspergillus* spp.colonization is an important prerequisite to subsequent infections,[2]particularlyin the lower respiratory airways.[3]

Conventional diagnostic tests such as blood cultures are not useful or practical for the diagnosis of Aspergillus spp. fungemia.[4] Further, non-culture–based techniques used in the past lack the sensitivity and specificity needed for the testing of immune compromised patients.[5] Additionally, established IA is difficult to treat and has a death rate of 80.0–90.0%,

which has driven researchers to develop more reliable and effective methods for its timely diagnosis.[6,7] Polymerase chain reaction (PCR) assays (conventional, nested, and real-time) have recently been developed for diagnosing fungal infections, especially quantitative real-time PCR (qPCR), which shows better potential for clinical application.[8,9]

Sensitivity and specificity of PCR in bronchoalveolar lavage fluid have been estimated to be 67.0%–100.0% and 55.0%–95.0%, respectively.[10]Until now, numerous studies have used target sequences from this region to detect Aspergillus DNA using PCR methods.[11,12]There are very few studies conducted in India emphasize on *Aspergillus* isolation from patients with complaints of lower respiratory tract infection (LRTI). However, a consensus is not available with regard to the specific target sequence, specimen type, extraction method, or PCR format and platform.

Geographical differences in the prevalence of different *Aspergillus* spp., especially in azole-resistant *A. fumigatus*have been observed among national and regional populations in the world, including India.[13] Although several studies on *A.fumigatus* in China and India have been reported,[13] studies focused on isolates of non-*A. fumigatus* spp. are limited, especially on cryptic *Aspergillus* species. Moreover, antifungal resistance in these reported isolates is rare.2

We hypothesized that Polymerase Chain Reaction can be used for rapid identification of *Aspergillus* genus in patients with Lower Respiratory Tract Infection which can help for an early diagnosis. Therefore, the aim of our study is to identify *Aspergillus* genus with conventional method and comparison with PCR method.We are targeting Inter Transcribed Spacer region (ITS). ITS is the spacer DNA situated between the small- subunit ribosomal RNA (rRNA) and large subunit rRNA genes in the chromosomes or the corresponding transcribed region in the polycistronicrRNA precursor transcript. The ITS region is the most widely sequenced DNA region in molecular ecology of fungi and recommended as the universal fungal barcode sequence.

2. MATERIAL AND METHODS

This prospective hospital based cross sectional observational study was carried out on 150 Sputum samples of patients having lower respiratory tract infections (LRTI) as defined the guidelines of the European Respiratory Society and the European Society for Clinical Microbiology and Infectious Disease on management of LRTI in adults visiting Department of TB and Chest, Index Hospital, Indore over a period of June 2018 June 2020. All samples were included in this study for incidence of *Aspergillus*species. All sample were collected from patients those were more than 18 years of age, acute episode of cough for 21 days, cough as the cardinal symptom, symptoms associated with lower respiratory tract infections such as sputum production,dyspnoea, wheeze, chest discomfort/pain. Samples collected from patients who were had active tuberculosis and atypical mycobacterial infections, and suffering from any malignancies were not included in this study.

Sample collection

First early morning 3-5 ml of sputum and whole blood sample were collected from patient .Direct Microscopy was performed in 10% Potassium hydroxide to see the presence of fungal elements . Sputum sample also homogenized and separated into two parts. One part of sputum was cultured on SDA with chloramphenicol slants and incubated for 3 to 5 days. Isolates were identified on the basis of microscopic and macroscopic morphological characteristics. [14]

Serum is separated from blood sample for the detection of AspergillusIgG and IgE antibodies by commercially available kit (Omega Diagnostics,Calbiotech) based on ELISA method of identification.

PCR specification

The second part of sputum was used for DNA extraction. DNA extraction is done by Qiagen Q Amp mini kit following manufacture's guidelines with some changes and stored under -20 degree C. PCR was done using open kit method using Taq DNA polymerase(0.15ul), PCR buffer(2.5ul), dNTPs(0.5ul), taqpol(0.15ul), 5xQ buffer(5ul) , DNAsample(3ul) with a set of ITS 5-4 primers with oligonucleotide sequence for Aspergillus genus (Eurofins, genomics) ITS5-5'GGAAGTAAAAGTCGTAACAAGG-3'andITS4-5'TCCTCCGCTTATTGATATGC 3'. PCR was standardised and concentrations of these components of PCR optimized with cycling conditions.Final volume of reaction mixture is achieved 25ul by adding 11.85ul nuclease free water[9] .Thermocycler was programmed for 40 cycles with initial denaturation at 95^0c for 5 min followed by denaturation at 95^0c for 3 sec, annealing at 48-48.5^0c for 30 sec, extension at 72^0c for 45 sec. Amplified PCR products were visualised under ultra-violet transluminator by 1.5% agrose gel electrophoresis at 70V for 30 min. The presence of unique band around 600bp similar with provided ladder was indicative of presence of Aspergillus spp. DNA in sputum sample of patients.

3. RESULTS AND DISCUSSION:

The mean age of the patients from those samples were collected was 43.4±15.9 years and the majority of the patients were males 100(66.7%). The bronchial asthma (27.3%) was the major disease followed by COPD (23.3%) and chronic bronchitis (13.3%). 10.0% KOH findings show 25.3% i.e. 38 positive cases of total 150 cases.Our findings were in accordance with Abood AL-Asadi ZH et al[15] where the ages of participants ranged from 10–70 years with a mean age of 34 years. The highest incidence 26(53.06%) was found in patients aged 30–40 years from the studied specimens followed by age groups 40–50 years and constitute 13(26.5), then (50–60) and (60–70) years old which recorded (10.27%, 7.54%) respectively while the lowest infection recorded in age group 20–30 years was 6.1% and in 10–20 years old which recorded 2(2.04) from the infected peoples. Shrimali GP et al[21] reported the highest number of patients were from 31-40 years of age and then gradually decrease in number in 21-30 and 41-50 years of age group.In the present study the majority of the studied samples were of males (66.7%) followed by females (33.3%). Our findings were comparable to the Sharma A et al[22] who reported that among the study population, male patients were more affected than females. According to Salah H et al[16] seventy Aspergillus strains were isolated from clinical specimens obtained from 67 patients including 40 males and 27 females. Shrimali GP et al[21]in their study found that out of the total 100 patients 79 (79.0%) were males and 21.0% were females. A male predominance is suggestive of the involvement in outdoor activity hence exposure to airborne pathogens. AboodAL-Asadi ZH et al[23]reported that the males were more susceptible to fungal infection, were recorded 35/53 (66.3%), compared to females were 18/53 (33.9%). In our study the patients were distributed according to the disease category and it was found that the majority of the cases were of Bronchial Asthma (27.3%) followed by COPD (23.3%) and Chronic Brochititis (13.3%). Our findings were similar to the study performed by Sharma A et al[22] who reported that of the 150 patients suffering from LRTI, 30.0% were clinically categorized as a chronic obstructive pulmonary disease (COPD), 22.0% bronchial asthma (BA), and 4.0% chronic bronchitis (CB). The remaining 44.0% patients had exaggerated symptoms of LRTI but could not be

categorized in any specific clinical groupAspergillusfumigatuswas found to be a major fungus associated with bronchial colonization in patients with lung disease[17].
Of 150 sputum samples, 23.3% (35/150) grew Aspergillus species, of which 11.3% (17/150) were Aspergillusfumigatus, 10.0% (15/150) Aspergillusflavus, and 2.0% (2/150) Aspergillusniger. [Table 1]Antifungal susceptibility of isolate aspergillus genus against itraconazole, caspofungin, and amphotericin was found sensitive in all patients except one who shows resistant for itraconazole inA.fumigatus. [Table 2]. In the present studyout of the total 150 patients 38 (25.3%) were found positive whereas 74.7% were found negative for respiratory disease. Abood AL-Asadi ZH et al[23]reported that out of hundred samples were subjected to 10% KOH examination, 35 (35%) of the samples showed positive result by 10% KOH examination which was near about similar to the present study. Sharma A et al[22] shows 13.0% sputum samples were positive for the presence of septate fungal hyphae of which 11.0% were culture positive. Two percent of sputum samples could not yield any growth on culture though were positive for KOH and 87.0% sputum samples were KOH negative. A study by Kurhade AM *et al*[18] revealed that 16.0% of respiratory specimens were KOH positive for *Aspergillus* spp.In the present study out of the 35 (35/150) positive sample for aspergilosis17 (11.3%) were found positive for A-fumigatus, 15 (10.0%) were found positive for A-flavus whereas only 2.0% were found positive for A-Niger isolate. Sharma A et al[22] in their study on molecular detection of *aspergillus* in sputum of patients with lower respiratory tract infections reported that of 150 sputum samples 19.0% (29/150) grew *Aspergillus* species, of which 11.0% (17/150) were *Aspergillusfumigatus*, 7.0% (11/150) *Aspergillusflavus*, and 0.6% (1/150) *Aspergillusniger*.In a study by Abood AL-Asadi ZH et al[23], the predominant Aspergillus species isolated from sputum was Aspergillusfumigatus (42.04%) while A. flavus, A. niger, and A. terreus were isolated from 11(20.08%), (13.2%) and 3(5.7%) patients respectively, also isolated Penicillium spp. at percentage 1(1.9%). The identification of A. fumigatus by using conventional methods which agreed with result of the same study conducted by Ellis et al[19] showed that the A. fumigatus colonies on Czapek-Dox agar appear a grey green color as a result of the conidia pigmentation. Salman AR et al[20] in their study on Isolation and Identification of Aspergillus spp. reported that Aspergillus spp. isolated as highly percentage of frequency (36.47%) where consist of Aspergillusfumigatus with (16.2%), Aspergillusniger (10.8), Aspergillusflavus (6.7%) and Aspergillusochrecious (2.7%). Aspergillusfumigatus has been reported as the most prevalent species causing IA in different parts of the world, including the United States, Europe and Brazil.[21]Hussein HM et al[18] reported that only 25(41.7%) out of 60 (100.0%) patients exhibited a growth of Aspergillusspeciesin culture. The most isolated species were A. fumigatus as 23(92.0%) followed by A. flavus as 2 (8.0%). Similar with studies done by Karthikeyan P et al[22], (2010) who mentioned that A. fumigatus was the most common species 69.0% of all fungal pathogens isolated from patients with chronic rhinosinusitis followed by A. flavus and A. nidulans in 7.0% each. In contrast to our study, Tashiro T et al[23]isolated 42.0% *Aspergillus* spp., in cases representing colonization, and the most common colonizing species was A. niger, followed by *Aspergillusversicolor, A. fumigatus, Aspergillusterreus, A. flavus, Aspergillussydowii,* and *Aspergillusnidulans*. Due to geographical diversity though predominance of *A. flavus* as the causative agent other spp. may predominate locally hence, leading to exposure and infection.
The findings of PCR test in all the studied patients and out of the total 150 patients 48 (32.0%) were found positive whereas 68.0% were found negative for respiratory disease. [Figure 1]In the present study the *Aspergillus*species were isolated from sputum samples was found to be 35 out of the total 150 samples (23.3%) by conventional method. In the similar study Hussein HM et al[24] in 2019 did a study on the evaluation of nested PCR for the

diagnosis of aspergillusrhinosinusitis in comparison with conventional methods and found that twenty five samples (41.7%) out of the 60 (100%) exhibited a growth of Aspergillus species. According to Grover S et al[25] incidence was 28.3% and according to Jain SK at al[26]incidence was 22.2%. 29% of 100 samples were aspergillus spp. according to Shrimali GP et al[27]. Our findings were also similar to the Sharma A et al[28] who reported 29 out of 150 sputum sample positive for aspergillusspecies.In this study the findings of PCR test in all the studied patients observed that out of the total 150 patients 48 (32.0%) were found positive whereas 68.0% were found negative for respiratory disease. In a study by Singh R et al[29], *Aspergillus* DNA was detected in 25.0% of sputum samples of suspected aspergillosis patients using nested PCR using specific primer sets (Nig, fmi, and Fla). In the study by Sharma A et al[22], PCR detected *Aspergillus* in sputum of 28.0% (43) LRTI patients.

In the present study the Sensitivity, specificity, of 10% KOH, and culture findings against PCR was found to be 68.7%, 95.1% respectively for KOH and 72.9% and 100.0% respectively for culture test. [Table 3 and 4]. Among 48(32.0%) PCR-positive sputum samples, 35 (23.3%) was also found to be culture positive for *Aspergillus* species. 0 (0.0%) sputum samples were found to be culture positive and PCR negative while 102 (68%) were culture as well as PCR negative.

The ITS region contains the variable elements that allow for sequence based identification according to Iwen et al(2002) otherwise PCR-based detection or identification systems for Aspergillus species based on using 18S or 28S rDNA as target DNA.however, the sequences in theses regions are conserved across a wide range of fungi, it is therefore difficult to design truly species specific primers. As reported in Henry et al ITS region is proven more useful for identification of fungal species .Kawamura et al also found high sensitivity of the nested PCR for the detection of AspergillusDNA in patients with pulmonary Aspergillosis with set of primers M5c and M6B .However we detectedAspergillus DNA in sputum sample using ITS4 and ITS 5 primers which found similar to Sharma.A et al. In our studyPCR was found to be helpful for detecting Aspergillus DNA in sputum sample in LRTI patients especially in Aspergilloma and ABPA which are non invasive form of Aspergillosis..In the present study, false-positive results of PCR may be attributed to the inability of PCR to differentiate between colonization and infection, also due to high susceptibility of contamination of PCR in laboratory.Our findings were concordance with Sharma A et al[22] who reported the Sensitivity and specificity of PCR to be 89.7% and 85.96% with culture as gold standard. The positive predictive value was 60.5% and a higher negative predictive value 97.21% was obtained ($P < 0.0001$). Singh R et al[33] also found sensitivity of nested PCR to be higher than culture and microscopy. In a study by Hussein MH et al[18], culture method is gold standard in comparison with other laboratory techniques for detection of Aspergillus species. The nested PCR have been reported the sensitivity as 68.0%, positive predictive value (PPV) 65.0%, specificity 74.0% and negative predictive value (NPV) 76.0%. Abood AL-Asadi ZH et al[23], statistical analysis revealed that the PCR to have a sensitivity of 95.1% in the detection of Aspergillusfumigatus in Aspergillosis cases. Polymerase chain reaction (PCR) is a rapid, specific, and sensitive method to detect Aspergillusfumigatus in aspergillosis cases of humans.According to Knutsen and Slavin[30] PCR for detecting *Aspergillus* spp., in sputum is more sensitive than culture in ABPA but needs to be interpreted with other clinical laboratory features. Positive culture of *Aspergillus* species although indicative but does not prove infection. Gold standard of myological/or histological evidence of invasion is not always feasible to obtain.

Conclusion

PCR helped in early detection of *Aspergillus* genus in LRTI patients where sputum cultures were negative. Aspergillus. fumigatus was the most dominant molds isolated from

sputum in aspergillosis patients. PCR method has a high degree of specificity for the identification of Aspergillus spp. Therefore, as a point of care, routine screening of their sputum samples for the presence of *Aspergillus* by PCR technique along with culture can be done. Gold standard of myological/or histological evidence of invasion is not always feasible to obtain.

Table 1: Fungal isolates findings

Fungal Isolate		**Frequency (n=150)**	**Percentage**
A.fumigatus	**Negative**	133	88.7
	Positive	17	11.3
A.flavus	**Negative**	135	90.0
	Positive	15	10.0
A.niger	**Negative**	147	98.0
	Positive	3	2.0

Table 2: Antifungal susceptibility of isolate aspergillus genus against itraconazole, caspofungin and amphotericin.

Isolate Aspergillus	**Antifungal**					
	Itraconazole		**Caspofungin**		**Amphotericin**	
	Sensitive	**Resistant**	**Sensitive**	**Resistant**	**Sensitive**	**Resistant**
A-fumigatus (n=17)	16	1	17	0	17	0
A-flavus (n=15)	15	0	15	0	15	0
A niger (n=3)	3	0	3	0	3	0

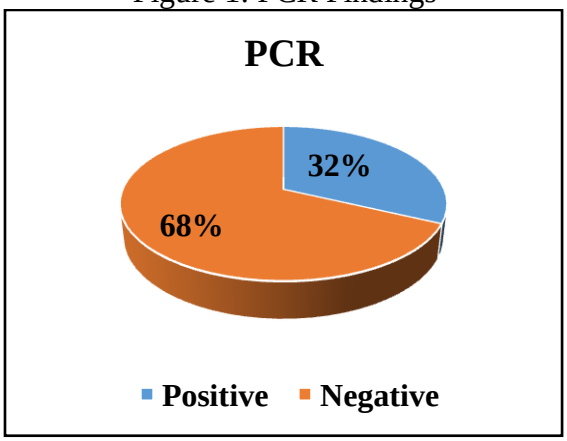
Table 3: Association of 10.0% KOH with PCR detection findings

		PCR Detection		**Total**
		Positive	**Negative**	
10.0% KOH	**Positive**	33 (22.0)	5 (3.3)	38 (25.3)
	Negative	15 (10.0)	97 (96.7)	112 (74.7)
Total		48 (32.0)	102 (68.0)	150 (100.0)
p-value		**<0.01**		

Table 4 Association of Culture and PCR detection findings

		PCR Detection		**Total**
		Positive	**Negative**	
Culture	**Positive**	35 (23.3)	0 (0.0)	35 (23.3)
	Negative	13 (8.7)	102 (68.0)	115 (76.7)
Total		48 (32.0)	102 (68.0)	150 (100.0)
p-value		**0.01**		

Figure 1: PCR Findings



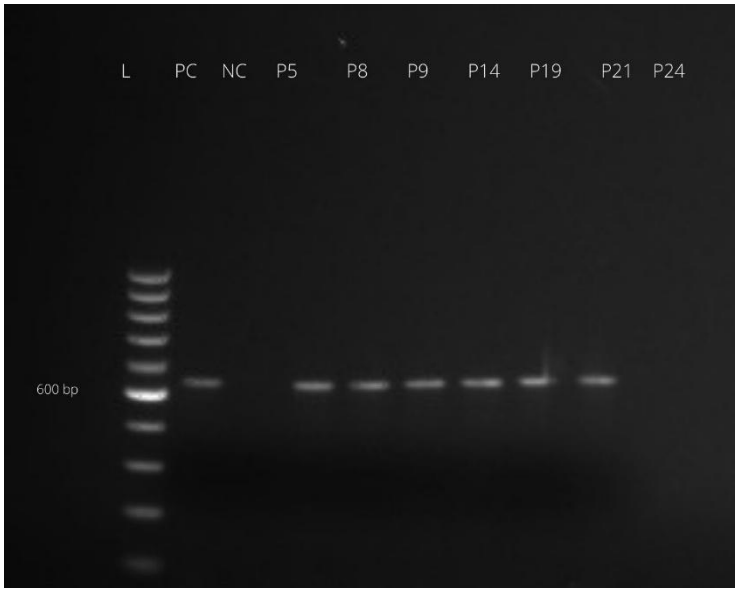


Figure 2: Visualization of amplified PCR products under UV trans illuminator on gel electrophoresis showing unique band around 600 bp of Aspergillus genus , NC : negative control ; PC: positive control ; L : base pair ladder , P5,P8,P9,P14,P19,P21, P24 were patient samples

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