

Comparison of Various Laboratory Detection Methods for Diagnosing Pulmonary Aspergillosis

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Abstract

Aspergillus is a mould which may lead to a variety of infectious, allergic diseases depending on the immune status or pulmonary structure of the host. Invasive pulmonary aspergillosis occurs primarily in patients with severe immunodeficiency. Early and rapid diagnosis of systemic fungal infections remain limited, despite intensive efforts by many investigators. Few clinical guidelines have been previously proposed for either diagnosis or management of Pulmonary Aspergillosis. The current study was undertaken for comparing various detection methods like conventional direct microscopy or histopathology, culture methods, immunological (GM ELISA) and molecular methods (PCR). To identify carcinoma cell types and to categorize Aspergillosis types histopathology was done. For direct fungal examination, culture, *Aspergillus* polymerase chain reaction (PCR), and galactomannan (GM) detection, bronchoalveolar lavage (BAL) fluids were collected. In this study microscopy was found to have low sensitivity of (70.5%) while culture had highest specificity (97.6%). GM assay showed a sensitivity of 100% and a specificity of 86.4% whereas PCR has an overall sensitivity of 100% and a specificity of 81.3%. Thus, we suggest that both BAL PCR and GM ELISA may be beneficial for use in early diagnosis of Aspergillosis, especially those patients who do not demonstrate radiological signs.

Keywords: Pulmonary Aspergillosis; Diagnosis; PCR; ELISA

Introduction

IPA was first described in 1953 [1]. Due to widespread use of chemotherapy and immunosuppressive agents, its incidence has increased over the past two decades [2]. Despite intensive efforts by many investigators, early and rapid diagnosis of systemic fungal infections remain limited. *Aspergillus* spp. when isolated from respiratory samples does not confirm it as the etiologic pathogen because airway colonization by *Aspergillus* spp. is a common feature in several chronic lung diseases. Only if *Aspergillus* spp. is repeatedly isolated and anti- *Aspergillus* antibodies and/or *Aspergillus* antigens in sera are detected, it points towards the etiologic agent of disease [3]. The only available techniques in most centers to diagnose IA are conventional direct microscopy, histopathology, and culture methods, due to the non-availability of galactomannan or beta-glucan tests assay in developing countries [4].

Bronchoscopy with Bronchoalveolar Lavage (BAL) is generally helpful in diagnosis of IPA especially in patients with diffuse lung involvement [5]. Generally, clinical and radiologic signs are insensitive and non-specific, and the sensitivity of fungal culture is low [5]. Histopathology plays a good diagnostic role in IPA, but the invasive nature of tissue biopsy collection discourages its use in thrombocytopenic patients. Thus, rapid and more sensitive diagnostic strategies for fungal infections including detection of antigen or DNA are being evaluated [6,7].

Galactomannan (GM), double sandwich ELISA (Platelia, Bio-rad) which incorporates the β 1-5 Galactofuranose specific EBA2 monoclonal antibody as both the acceptor and the detector for Galactomannan, a polysaccharide cell-wall component that is released by *Aspergillus* during growth, has the most promise among the many tests that have been developed for detection [7]. In the Revised Criteria of EORTC/MSG for probable invasive Aspergillosis, the Galactomannan antigen detection has also been included. Although, the detection of nucleic acid is not included in the criteria, as presently, there are no validated or standardized methods [8]. While GM cannot identify infecting

Aspergillus species, PCR could be tailored to the species level and may infer general antifungal susceptibility pattern [5].

Materials and Methods

This study was conducted on patients admitted in the wards or attending the outpatient department of T.B. and Respiratory Diseases, Jawaharlal Nehru Medical College and Hospital, AMU, Aligarh, for a period of one and half year. Study group comprised of patients suspected of lung carcinoma and chronic lung diseases like interstitial lung disease, chronic obstructive pulmonary disease etc. Bronchoscopy was performed for confirmation of clinical diagnosis in around 60 patients. Age and sex matched healthy controls were also included in the study with no evidence of any chronic lung disease.

A detailed clinical history was recorded for each patient especially regarding duration of illness, occupation (especially any exposure to grains), history of smoking/gutka chewing, dietary history and others like: Signs and symptoms, broad spectrum antibiotics and corticosteroid therapy.

In a clean sterile vial, Bronchoalveolar lavage (BAL) was collected, by fiberoptic bronchoscopy. Each BAL specimen was divided in four parts for direct microscopy, culture, galactomannan and PCR.

Each specimen of bronchoalveolar lavage was subjected to various laboratory procedures for isolation and identification of *Aspergillus* spp.

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Direct microscopy

BAL was homogenized using vortex and subjected to direct mount and lactophenol cotton blue (LPCB) mount. One drop of homogenized BAL and one drop of lactophenol cotton blue was taken at two ends of a clean and sterile glass slide. Then a loopful of BAL was emulsified in LCB. Separate coverslips were placed on each of the drops and examined under microscope (10X and 40X) to look for fungal elements like fungal hyphae, heads and spores of *Aspergillus* species.

Culture

Subsequently BAL was streaked on two sets of Sabouraud dextrose agar (SDA)- Plain and SDA containing chloramphenicol (0.05 mg/ml) and on two sets of Czapek dox agar. One set of both the media was incubated at 25°C and the other at 37°C.

All the culture media were examined for fungal growth daily during the first week and thereafter every 2-3 days up to 3 weeks before reporting the specimen as negative for fungus.

Tease mount preparation and microslide culture was done for further characterization of fungus.

Galactomannan antigen:

was detected in bronchoalveolar lavage (BAL) and serum samples using PLATELIA™ *ASPERGILLUS* Ag ELISA kit (Bio Rad, France).

This test, when used in conjunction with other diagnostic procedures such as microbiological culture, histological examination of biopsy samples and radiographic evidence can be used as an aid in the diagnosis of Invasive Aspergillosis.

Detection of *Aspergillus*-DNA in BAL-Polymerase Chain Reaction (PCR)

Preparation of template for PCR: DNA was extracted according to the method described by Lee and Taylor with minor modifications. To 100 µl of the homogenized BAL, 400 µl of lysis buffer containing 50 mM Tris-HCl (pH 7.2), 50 mM EDTA, 3% SDS, and 1% β-mercaptoethanol was added. This mixture was vortexed for 15 sec, incubated at 65°C or 1 hr and then boiled for 10 min. An equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) was added to the mixture and vortexed for 10 sec. Mixture was centrifuged at 14,000 for 15 min and to the aqueous phase, 10 µl of 3 M Sodium Acetate (pH 5.2) and 5.4 volume of the isopropanol were added. The tubes were inverted to mix gently, and the DNA was precipitated at -20°C for 1 h. The tubes were microcentrifuged again at 14,000 rpm for 10 min and the supernatant was decanted. The DNA-pellets were washed with 70% ethanol, dried and resuspended in 25 µl of sterile Millipore water. 10 µl of DNA template was used in PCR reaction.

PCR assay: Primers used in the study (5'- GAA AGG TCA GGT GTT CGA GTC AC 3' and 5'- CTT TGG TTG CGG GTT TAG GGA TT 3') amplified 135 base pairs (bp) of *A. fumigatus*, 118 bp of *A. flavus*, and 180 bp of *A. niger* [6].

Results

Invasive aspergillosis is an increasingly common and often fatal opportunistic fungal infection. However, the clinical symptoms and radiological signs of IA are frequently non-specific and occur late in the course of disease, making early diagnosis virtually impossible. Several specific diagnostic methods have been proposed to help establish an early diagnosis, including, for example, high resolution

computed tomography (CT) as well as detection of galactomannan antigen, fungal DNA by polymerase chain reaction or beta-D-glucan [9]. The use of high resolution CT could result in earlier diagnosis, but CT signs are non-specific for Aspergillosis [10]. The Galactomannan Enzyme Immuno Assay demonstrates proven reliability in patients with hematological malignancy; however, its sensitivity varies from 44-90% [11,12]. PCR has also been evaluated and appears promising as a potential diagnostic modality but is not commercially available for diagnosis of Aspergillosis [13].

In the present study, the patients with chronic lung diseases and lung cancer were studied for Aspergillosis and effort was made to study antifungal susceptibility pattern and to detect *Aspergillus* infection in BAL and sera by galactomannan antigen and PCR.

In our study *Aspergillus* spp. was isolated from BAL in 18 (30%) of the cases. Study conducted by Takayoshi Tashir et al. on immunocompetent patients in pneumology ward, Japan reported that 45% of their patients colonized with *Aspergillus* spp., displayed no clinical symptoms of Aspergillosis, while 55% had some form of pulmonary Aspergillosis [3].

All BAL and serum samples for galactomannan antigen were tested as well. In 38.3% of samples, antigen was detected. Both BAL and Sera were positive for GM antigen in 23.3% of cases. In 15% of the patients galactomannan was positive only in BAL and their sera were negative for the antigen. None of the case showed GM antigen in serum alone.

In patients of lung carcinoma, galactomannan antigen was positive in 37.5% of BAL and 25% of serum samples. Here again, 18.7% patients only BAL showed the presence of galactomannan antigen which is similar to the 36.2% galactomannan positivity in BAL of lung carcinoma patients as reported by Shahid et al. [14].

In this study, culture for *Aspergillus* was positive in 30% of cases which was less as compared to galactomannan antigen in both (38.3%) BAL and sera. 18 patients showed the growth of *Aspergillus* on culture while 23 patients were positive for galactomannan antigen. Hence culture positivity was seen in 78.2% of antigen positive patients as shown in Table 1.

Meersseman et al. cultured BAL specimens in intensive care unit patients for aspergillosis. Culture or direct examination was positive for *Aspergillus* in 60% of cases, while serum antigen was positive in 42%. However, antigen was detected in BAL in 88%. Specificity for antigen detection in BAL (87%) was higher than for culture or direct examination (70%) [15].

Our findings were like that of Meersseman et al. as BAL antigen was positive in more cases (38.3%) as compared to antigen in serum or culture and microscopic findings. However, culture for *Aspergillus* was positive in 30% of cases which was slightly more as compared to antigen in serum (23.3%).

As per our findings and other studies quoted earlier, we consider antigen detection to be better indicator of invasive Aspergillosis as compared to culture and direct microscopy since galactomannan comes predominantly from the hyphae and to a lesser extent from the conidia. A positive GM result in a neutropenic patient frequently triggers antifungal therapy, as a positive GM result has been reported to appear before clinical signs [16]. Presence of galactomannan in the BAL fluid seems to be a better diagnostic indicator for hyphal growth than routine mycological culture.

Aspergillus DNA is another possible biological marker for the diagnosis of IA [17]. Thus, polymerase chain reaction (PCR) was done

Disease	Culture for Aspergillus		Galactomannan antigen			
	Positive	Negative	Total no. of cases positive for galactomannan antigen (%)	Only BAL positive (%)	Both BAL and serum positive (%)	Both BAL and serum negative (%)
Lung carcinoma (n=40)	13(32.5)	27(67.5)	15(37.5)	5(12.5)	10(25)	25(62.5)
Secondaries in lung (n=16)	4(25)	12(75)	6(37.5)	3(18.7)	4(25)	10(62.5)
Tuberculosis (n=2)	1(50)	1(50)	1(50)	0	0	1(50)
COPD (n=1)	0	1(100)	0	0	0	1(100)
Sarcoidosis (n=1)	0	1(100)	1(100)	1(100)	0	0
Total (n=60)	18(30)	42(70)	23(38.3)	9(15)	14(23.3)	37(61.6)

Table 1: Culture findings and galactomannan antigen in relation to clinical diseases.

Aspergillosis type		Direct microscopy Positivity/ HPE positive	Culture for aspergillosis Positivity	Samples positive for galactomannan antigen			CT scan findings positive for Aspergillus
				BAL only	Serum only	Both BAL and serum	
IPA (n=21)	Proven IPA (n=8)	7	8	2	0	6	8
	Probable IPA (n=9)	5	9	2	0	7	6
	Possible IPA (n=4)	0	1	3	0	1	2
Non-IPA (n=39)		0	0	2	0	0	3
Total (n=60)		12	18	9	0	14	19

*HPE- Histo-pathological examination

Table 2: Classification of patients according to Aspergillosis type (Ben De Pau et al.).

Aspergillosis Type (n)	Direct Microscopy/HPE (%)	Culture (%)	Serology Galactomannan Antigen detection (%)	PCR (%)
Proven IPA (n=8)	7 (87.5)	8 (100)	8 (100)	8 (100)
Probable IPA (n=9)	5 (55.5)	9 (100)	9 (100)	9 (100)
Possible IPA (n=4)	0	1 (25)	4 (100)	4 (100)
Non-IPA (n=39)	0	0	2 (5.1)	4 (10.2)
Total (n=60)	12 (20)	18 (30)	23 (38.3)	25 (41.6)
Sensitivity	70.5%	100%	100%	100%
Specificity	89.5%	97.6%	86.4%	81.3%

Table 3: Detection of Aspergillus by various Conventional, Serological and Molecular methods.

for detection of *Aspergillus* DNA in BAL fluid and serum. However, PCR in BAL fluid and serum has not been accepted as a diagnostic criterion for IPA, because of divergent results due to the lack of standardization [2,18]. BAL-PCR is an important modality along with ELISA for early diagnosis of invasive Aspergillosis as compared with the conventional methods of detection.

PCR was also performed. The findings for *Aspergillus* spp. in relation to clinical disease were seen. It was observed that 20/40 (50%) cases of lung carcinoma and 4/16 (25%) cases of secondaries in lung were detected by PCR for *Aspergillus* spp.

In our study, *Aspergillus* DNA was detected in 38.3% (23/60) cases. In all the proven, probable IPA cases, PCR was positive, while only 2/4 possible cases of IPA and 4/39 (10.2%) cases of non-IPA were PCR positive as shown in Table 2. Amongst the control patients, all were negative for PCR tests. In the present study, the PCR always confirmed

the culture results except for the 4 culture-negative BAL fluids in non-IPA patients. This is due to high sensitivity of PCR which could detect even small amount of genomic DNA of *Aspergillus*.

PCR is another way to diagnose IPA. Our study showed that PCR has an overall sensitivity of 100% and a specificity of 81.3% as shown in Table 3. According to Hizel et al. PCR in BAL fluid had an estimated sensitivity of 67-100% and specificity of 55-95% for IPA. Our findings are in concordance with those of Hizel et al. [19].

Discussion

Indeed, one of the main points of our study is that a PCR-positive result, when obtained on the first GM-positive serum sample from patients at risk for IA, was associated with a poor prognosis. Clinical and radiological signs, also associated with a poor prognosis, have been reported to appear after (median, 6 days) the first GM result in more

than half of the cases [20]. Thus, in decision making, a PCR-positive result could reinforce the decision to initiate antifungal therapy, with the understanding that the therapy could be stopped if diagnosis of IA was not confirmed. In contrast, a negative PCR result could argue in favor of postponing costly treatment with potential side effects until additional arguments favoring therapy were presented. Thus, PCR assay with BAL fluid specimens seems to be a promising method for diagnosis of IPA, especially when used in association with the GM detection test [21].

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