Distribution of Galactomannan Antigen in BAL and Serum Samples Among Aspergillus Isolates and Its Correlation with Culture among Immunocompetent and Immunocompromised Patients in Aligarh Region, a North India Town

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ABSTRACT

Background: Aspergillosis is one of the most common fungal infection among both general patients and immunocompromised individuals.

Methods: The study was carried out on 2 groups of patients: immunocompetent and immunocompromised comprising of a total of 90 patients. Microscopy, culture, identification of isolates was done and some specialised tests on serum and BAL for antigen detection were performed.

Result: Most of the patients i.e., 47 (31.3%) were between 31-40 years with a mean age of 32.5 years. The male to female ratio was 1.8:1. Galactomannan antigen was positive in 13 (28.9%) BAL samples and in 15 (33.3%) serum samples wherein the 45 BAL samples represented immunocompetent cases and 45 serum samples represented immunocompromised cases. Sensitivity and specificity of GM assay in BAL samples were found to be 100% and 97% respectively. On the other hand, sensitivity and specificity in serum samples were found to be 94.1% and 100% respectively. All patients were categorized into 4 categories as proven IPA, probable IPA, possible IPA and non-IPA. 22 (24.4%) patients were of proven invasive pulmonary aspergillosis (IPA), 7 (7.8%) of probable IPA, 2 (2.2%) belonged to possible IPA while 59 (65.5%) were of non IPA. All of the cases of proven IPA were positive on culture.

Conclusion: GM assay in BAL is very sensitive and specific marker for Aspergillosis while in case of serum samples it is somewhat lesser sensitive.

Keywords: Galactomannan Antigen, Bronchoalveolar Lavage, Immunocompetent And Immunocompromised.

Introduction

Lungs are vulnerable organs for fungal infections as they are the initial portal of entry for fungi causing deep mycoses. In immunocompetent patients, an aspergilloma is the most common pattern of pulmonary aspergillosis and usually develops in a pre-existing cavity, bulla, or cyst. Fungal respiratory infections generate concern in the expanding population of immunosuppressed patients. Opportunistic Infections (OI) are caused either by organisms of low or no virulence which are non-pathogenic in individuals with an intact immune system, or by known pathogens which present in a different way than usual in immunodeficient individuals, e.g. in the form of increased virulence, recurrence, multidrug resistance or atypical presentation like Aspergillus spp. and Candida spp. Various mycoses form the bulk of opportunistic infections in AIDS patients and are increasing in the form of an epidemic parallel to the AIDS epidemic. Among the various opportunistic infections, respiratory infections account for up to 70% of AIDS defining illnesses. Besides the most prevalent and well-known fungal pathogens such as Candida albicans and Aspergillus fumigatus, a large number of new emerging pathogens have been described.

Early detection and identification of Invasive Fungal Infection (IFI) have been shown to improve prognosis, but available testing modalities may not offer rapid results. Culture, direct examination, special stains, and histopathology have been the mainstay of laboratory diagnosis. For Aspergillus species antigen, galactomannan assay findings may be positive in the blood very early prior to clinical suspicion of invasive fungal infection and may be of use in monitoring and pre-emptive treatment in high-risk populations.

The need of the hour is to undertake more studies on fungal diagnostic technique especially in a country with a rising population like ours where both the rural and urban masses are potentially at risk.

Keeping the above burning issues and constraints in perspective, we undertook this study.
Materials and Methods

The study was carried out on 2 groups of patients: immunocompetent and immunocompromised comprising of a total of 90 patients. These patients were attending the outpatient department or admitted to under The Department of T.B. and Respiratory diseases, and the Antiretroviral clinic at Jawaharlal Nehru Medical College and Hospital, AMU. The period of study was from January 2015 to July 2016.

Selection of Cases: Study group and design: 150 patients were divided amongst 2 subgroups:

i. Immunocompetent – patients with clinical suspicion of lung carcinoma and chronic lung diseases like interstitial lung disease, chronic obstructive pulmonary disease etc.

ii. Immunocompromised patients – patients with weakened immune systems i.e, with significant neutropenia <500 neutrophils/µl for longer than 10 days. These include AIDS, cancer and transplant patients who are taking corticosteroids, certain immunosuppressive drugs; and those with inherited diseases that affect the immune system (e.g., congenital agammaglobulinemia, congenital IgA deficiency). Cases were recruited from the outpatient departments, wards, Intensive Care Units (ICU), Antiretroviral treatment clinic, J. N. Medical College Hospital, A.M.U., Aligarh.

Collection of specimens

i. Bronchoalveolar lavage- Bronchoalveolar lavage was collected in a clean sterile vial by fibreoptic bronchoscopy after taking written informed consent. The bronchoscope was inserted through the endotracheal tube and wedged in a subsegmental bronchus. Five 20mL sterile saline aliquots at room temperature were infused and manually aspirated with a 20-mL volume syringe. The first aliquot was discarded, and the others were pooled and immediately sent to the laboratory. Pulse oximetry, electrocardiogram, and ventilatory parameters were monitored throughout the procedure.

ii. Blood - 5 ml blood was collected by venepuncture with all aseptic precautions in sterile plain vial. The vein from which the blood was withdrawn was chosen before the skin was disinfected. If the patient had an existing IV line, blood was withdrawn below the existing IV line.

Processing of specimens in the microbiological laboratory

i. Blood
   - Serum was used for HIV testing for the confirmation of HIV status.
   - fungal culture in Biphasic brain heart infusion Agar/broth.
   - Serum was used for antigen detecetion test for Cryptococcus by latex agglutination test using ‘Cryptococcal antigen latex agglutination test’ (Meridian bioscience, Europe).
   - Serum was used to detect the Aspergillus galactomannan antigen by ‘Platelia Aspergillus EIA’ (Bio-Rad, Germany).
   - Estimation of CD4 cell counts.

ii. Sputum (Expectorated and induced): Sterile glass beads were added to the sputum sample and vortexed briefly, equal volumes of freshly prepared sodium citrate (2.94%) and 0.5% N-Acetyl L-Cysteine added to specimen and vortexed again for 10-30 seconds depending upon the consistency, the mixture was diluted in phosphate buffer by adding double the volume and centrifuged at 1000g for 15 minutes.

iii. Endotracheal aspirate: Endotracheal aspirate samples were considered valid for culture if < 10 squamous epithelial cells and > 25 neutrophils were present.

BAL and Pleural fluid: They were directly inoculated for the respective tests.

All of the respiratory samples were subjected to the following tests:

a) CULTURE: According to the standard guidelines. [16]

b) ANTIGEN DETECTION: Aspergillus antigen detection by Platelia (Only in BAL samples)

Characterization of fungal isolates obtained from culture of various clinical specimens

After initial inoculation and incubation, all culture media were examined for fungal growth daily during the first week and on alternate days thereafter up to 3 weeks. The isolates were identified on macroscopic and microscopic morphological characteristics using standard techniques described in Medical mycology.[17]

ANTIGEN DETECTION IN RESPIRATORY SAMPLES AND SERUM

i. EIA for the detection of Aspergillus antigen in serum and BAL samples

The Platelia TM aspergillus EIA (BioRad, Germany) is an immunoenzymatic sandwich microplate assay for
the detection of aspergillus galactomannan antigen. In our study, 90 samples were tested, 45 each of BAL and serum.

**Result**

Galactomannan antigen was positive in 13 (28.9%) BAL samples and in 15 (33.3%) serum samples wherein the 45 BAL samples represented immunocompetent cases and 45 serum samples represented immunocompromised cases. Sensitivity and specificity of GM assay in BAL samples were found to be 100% and 97% respectively. On the other hand, sensitivity and specificity in serum samples were found to be 94.1% and 100% respectively. Table:1

In patients of HIV, 10 (33.3%) were positive for Aspergillus culture and galactomannan antigen in serum. Cases of pulmonary tuberculosis showed a positivity of 1 (50%) on culture and the same for galactomannan antigen. In patients with lung carcinoma 10 (26.3%) were positive for culture while 11 (29%) were positive for galactomannan antigen. 21% of these patients had galactomannan in BAL while 7.9% of them had antigen in serum. In patients with secondaries in lung 5(41.7.3%) were positive for culture and 4(33.3%) for galactomannan antigen i.e., 25% in BAL and 8.3% in serum. In cases of COPD 1 (20%) was culture positive and positive for galactomannan antigen in BAL as well. In cases of pneumonia 1 (50%) was positive for culture and galactomannan antigen in serum. Figure:2, Table:2.

All patients were categorized into 4 categories as proven IPA, probable IPA, possible IPA and non-IPA. 22 (24.4%) patients were of proven invasive pulmonary aspergillosis (IPA), 7 (7.8%) of probable IPA, 2 (2.2%) belonged to possible IPA while 59 (65.5%) were of non IPA. Table:3

All of the cases of proven IPA were positive on culture. 20 (91%) of them showed fungal elements for Aspergillus on direct microscopy and 16 (72.7%) showed histopathological findings of Aspergillus. 12 of these patients were positive for GM in BAL and 10 in serum. Also, 20 cases showed radiological findings positive for pulmonary Aspergillosis.

In all 7 cases of probable IPA, the patients were positive on culture except for 1. 6 (85.7%) showed fungal elements on direct microscopy, 1 of them had GM in BAL and 5 had antigen in serum. Radiological findings were positive only in 4 out of 7 patients.

In case of possible IPA none of the 2 patients were positive for culture or GM antigen. 2 showed positive findings on radiology. Among non IPA none of the 59 cases were positive for Aspergillus culture nor showed ant fungal elements on direct microscopy. 4 of the cases showed radiological findings conclusive of Aspergillosis. Table:4

| Clinical cases       | BAL samples positive for galactomannan antigen (%) (N=45) | Serum samples positive (%) (N=45) |
|----------------------|----------------------------------------------------------|----------------------------------|
| HIV (n=30)           | 0                                                        | 10(33.3)                         |
| Pulmonary Tuberculosis (n=2) | 1(50)                                                     | 0                                |
| Lung mass            |                                                          |                                  |
| Lung carcinoma (n=38) | 8(21.0)                                                   | 3(7.9)                           |
|                      |                                                          | 3(25)                            |
|                      |                                                          | 1(8.3)                           |
| Secondaries (n=12)   |                                                          |                                  |
| COPD (n=5)           | 1(20)                                                     | 0                                |
| ARDS (n=1)           | 0                                                         | 0                                |
| Pneumonia (n=2)      | 0                                                         | 1(50)                            |
| Total (n=90)         | 13(28.9)                                                  | 15(33.3)                         |

| Clinical cases | Culture for aspergillus | Galactomannan Antigen |
|----------------|-------------------------|------------------------|
|                | Positive | Negative | BAL samples positive for galactomannan antigen (%) | Serum samples positive |
| HIV (n=30)     | 10(33.3) | 20(66.6) | 0 | 10(33.3) |
### Clinical cases

| Clinical cases       | Culture for aspergillus | Galactomannan Antigen |
|----------------------|-------------------------|------------------------|
|                      | Positive | Negative | BAL samples positive for galactomannan antigen (%) | Serum samples positive |
| Pulmonary Tuberculosis (n=2) | 1(50) | 1(50) | 1(50) | 0 |
| Lung mass Carcinoma (n=38) Secondaries (n=12) | 10(26.3) | 27(71) | 8(21.0) | 3(25) | 3(7.9) | 1(8.3) |
| COPD (n=5) | 1(20) | 4(80) | 1(20) | 0 |
| ARDS (n=1) | 0 | 1(100) | 0 | 0 |
| Pneumonia (n=2) | 1(50) | 1(50) | 0 | 1(50) |
| Total (n=90) | 28(31.1) | 62(68.9) | 13(14.3) | 15(33.3) |

Table: 3 Classification of patients according to Aspergillosis type (Ben De Pau et al., 2008).35.

| Aspergillosis type | Direct microscopy/ HPE positive | Culture for aspergillus Positivity | Galactomannan antigen positivity | CT scan findings positive for Aspergillus |
|--------------------|---------------------------------|-----------------------------------|---------------------------------|------------------------------------------|
| IPA (n=31)         | Proven IPA (n=22) | 20 | 22 | 12 | 10 | 20 |
| IPA (n=7)          | Probable IPA (n=7) | 7 | 6 | 1 | 5 | 4 |
| IPA (n=2)          | Possible IPA (n=2) | 0 | 0 | 0 | 0 | 2 |
| Non IPA (n=59)     | 0 | 0 | 0 | 0 | 4 |
| Total (n=90)       | 27 | 28 | 13 | 15 | 30 |

Table: 4 Correlation of culture positivity and galactomannan

| Galactomannan | No. of cases positive for Aspergillus culture | No. of cases negative for Aspergillus culture |
|---------------|---------------------------------------------|---------------------------------------------|
| Positive (n=28) | BAL(13) | 12 | 1 |
|                | Serum(15) | 15 | 0 |
| Negative (n=61) | BAL(33) | 0 | 32 |
|                | Serum(30) | 1 | 29 |
| Total (n=90) | 28 | 62 |
Discussion

Pulmonary disease caused by Aspergillus spp., mainly *A. fumigatus*, presents with a spectrum of clinical syndromes in the lung. \[18\] Diagnosis of IA is challenging, because clinical and radiologic signs are very insensitive or nonspecific. Employment of new diagnostic tests may allow a targeted therapeutic approach and more accurate identification of high-risk patients. \[19\] The current doubledirect sandwich enzyme-linked immunosorbent assay (ELISA) (Platelia Aspergillus, Bio-Rad Laboratories) uses the galactofuranose-specific rat monoclonal antibody EB-A2 for both capture and detection of GM, resulting in a lower threshold of detection. \[20\]

In the present study, GM assay was conducted in both BAL and serum samples, 45 each. BAL was taken from immunocompetent patients and serum from the immunocompromised. This was because reports have shown that in BAL samples the test showed a high sensitivity among patients who have non hematologic cancer (solid-organ transplant recipients, chronic obstructive pulmonary disease, patients in the intensive care unit (ICU)), \[21-25\] whereas the sensitivity of the test in serum was particularly low in this subgroup. These differences may be explained by a lower degree of angio invasion in these patients who are still capable of having a local inflammatory reaction confining the disease to the lung.

BAL positivity for GM was seen in 13(28.9%) samples and culture of same samples was positive in 12 (26.6%) patients. False positive results can be caused by intake of β-lactam antibiotics, blood transfusions, or blood-derived products, sodium gluconate (contained in Plasma-Lyte solution and some food products) or other food additives and cross-reactivity with other fungi. \[22-24\] It is not possible to exclude the involvement of other molds such as Fusarium, Zygomyces, and dematiaceous fungi. \[18\] Therefore, galactomannan detection does not remove the need for careful microbiological and clinical evaluations. \[18\] History of intake of steroids and antibiotics was present in the single case negative for culture and positive for GM.

In serum samples, GM was positive in 15(33.3%), the cultures of which showed positive isolates in 16(35.5%) samples. Higher positivity in serum samples as compared to BAL samples can be attributed to the pool of samples representing immunocompromised patients.

All patients were categorized into 1 of 4 categories based mainly on the criteria of the European Organization for Research and Treatment of Cancer/Mycoses Study Group (EORTC/MSG) as described by Ben De Pauw et al., as proven IPA, probable IPA, possible IPA and non-IPA. 22 (24.4%) patients were of proven IPA, 7 (7.8%) of probable IPA, 2 (2.2%) belonged to possible IPA while 59 (65.5%) were of non IPA. \[35\]

All of the cases of proven IPA were positive for culture. 20 (91%) of them showed fungal elements for Aspergillus on direct microscopy and 16 (72.7%) showed histopathological findings of Aspergillus. 12 of these patients were positive for GM in BAL and 10 in serum. Also, 20 cases showed radiological findings positive for pulmonary Aspergillosis.

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antigen in serum. Radiological findings were positive only in 4 out of 7 patients.

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In a study conducted by Ali S et al., 20% patients were found to be of proven IPA, 32.5% of probable IPA and 17.5% of possible IPA and 30% of their patients did not meet any of the criteria for IPA.[36]

Therefore, GM assay in BAL showed a sensitivity of 100% and a specificity of 97%. A meta-analysis study was undertaken to assess the accuracy of GM assay for diagnosing IPA. 27 studies from 1996 to 2005 were included, and cases were diagnosed with IPA according to EORTC/MSG criteria. Overall, the assay had a sensitivity of 71% and specificity of 89% for proven cases of invasive Aspergillosis.[37] Our specificity of GM assay is similar to that analysed by Pfeiffer et al., although our study showed a higher sensitivity.

In serum samples, however a lower sensitivity of 94.1% was observed. According to Lahmer T et al., (2016)38, a lower sensitivity of 35% and a specificity of 70% were observed in serum samples as compared to a sensitivity of 70% and a specificity of 94% using BAL samples in the same critically ill patients.

**Conclusion**

GM assay in BAL is very sensitive and specific marker for Aspergillosis while in case of serum samples it is somewhat lesser sensitive. The need of the hour is to undertake more studies on fungal diagnostic technique especially in a country with a rising population like ours where both the rural and urban masses are potentially at risk.

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