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Original Article

Pulmonary aspergillosis as opportunistic mycoses in a cohort of human immunodeficiency virus-infected patients: Report from a tertiary care hospital in North India

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ABSTRACT

Objective: The incidence of pulmonary aspergillosis in human immunodeficiency virus (HIV)-infected persons is rising. This study was designed to determine the prevalence of pulmonary aspergillosis in a cohort of HIV-positive patients (n = 71) presenting with lower respiratory tract infection at a tertiary care medical center in India.

Methods: Sputum samples were collected, and potassium hydroxide mount, cultural characteristics, and lactophenol cotton blue preparations were employed to aid in the identification of *Aspergillus* species. In addition, serum galactomannan antigen testing was also performed.

Results: Pulmonary aspergillosis was diagnosed in 7 patients, five of whom showed a positive antigenemia indicating invasive form of disease. The prevalence of pulmonary aspergillosis was highest in individuals 21-40 years of age (13.3%). The gender-wise prevalence of pulmonary aspergillosis was 18.7% and 7.7% in females and males, respectively. The common chest radiographic findings noted in patients with pulmonary aspergillosis included a normal chest radiograph in 3 (42.8%), infiltrates in 2 (28.6%), and pleural effusion in 2 (28.6%). The common *Aspergillus* species recovered from sputa of these patients were *Aspergillus flavus* (4; 57.1%); *Aspergillus fumigatus* (2; 28.6%), and *Aspergillus niger* (1; 14.3%). A predisposing lung condition in the form of pulmonary tuberculosis was identified in 2; *Pneumocystis carinii* pneumonia in 2 and a dual tubercular and *P. carinii* infection in one. The mean CD4 count of these patients was 155.86 ± 119.33 cells/µl (median = 117 cells/µl; range = 18-329 cells/µl).

Conclusion: Our findings suggest that *Aspergillus* species be considered possible etiological agents in HIV-positive patients with pulmonary infection.

Keywords: Acquired immunodeficiency syndrome, *Aspergillus*, human immunodeficiency virus, galactomannan, pulmonary

INTRODUCTION

Acquired immunodeficiency syndrome (AIDS) caused by human immunodeficiency virus (HIV) is known to lead to profound immunosuppression. In the majority of the HIV-positive patients, the cause of morbidity and mortality is not the virus itself but the opportunistic infections that result from an immunocompromised state.[1] According to the Centre for Disease Control and Prevention (CDC), more than 20% of the AIDS-defining diseases reported, are fungal diseases affecting any anatomic site.[2] A common opportunistic invasive mycosis encountered is invasive aspergillosis. This clinical entity is generally seen in advanced stages of AIDS and most commonly involves the lung.[3,4]

Although infection by *Aspergillus* species was initially included by CDC in the list of AIDS-defining illnesses, it was deleted in the subsequent revisions, the primary reason for this was that aspergillosis is a relatively uncommon and unusual entity in AIDS patients.[5] This can be explained by the fact that while HIV infection is characterized by depletion of CD4 population of T-cells, it is the depletion of neutrophils and macrophages that play a central role in the pathogenesis of aspergillosis.[6] The disease is, therefore, more commonly seen in disease states associated with neutropenia or a defective cell-mediated immune response such as patients on cytotoxic chemotherapeutic agents, immunosuppressants, high-dose/prolonged steroids, broad-spectrum antimicrobials, and in patients with hematological malignancies.[7,8] However, reports have suggested a resurgence and a possible rising incidence of pulmonary aspergillosis in patients with HIV/AIDS.[3,9,10] HIV-positive patients with pulmonary aspergillosis, particularly the

invasive form, generally have very low CD4 counts and often other risk factors are concomitantly present.[11]

The relative lack of any pathognomic sign or symptom, the frequent absence of roentgenographic features in immunocompromised patients as well as the non-specific nature of computerized tomography findings often preclude an early diagnosis of pulmonary aspergillosis. Moreover, the prognosis of HIV-associated pulmonary aspergillosis, particularly the invasive form is generally poor, with a median survival of 3 months following diagnosis.[12]

The poor prognosis of pulmonary aspergillosis in AIDS also requires that to improve the survival of patients, diagnosis is made both as rapidly and at a stage as early as possible so that appropriate and effective antifungal regimen can be instituted promptly. The diagnostic yield of conventional diagnostic modalities including culture and histopathology is generally low.[13] This calls for non-culture based techniques for diagnosis such as detection of *Aspergillus* galactomannan antigen and molecular methods such as polymerase chain reaction. However, molecular assays are not available routinely in most laboratories and are therefore not considered standard for diagnosis.[14] In contrast, assays for detection of galactomannan are widely available commercially.[15]

Information on HIV/AIDS associated pulmonary aspergillosis is generally scarce especially from an Indian perspective, and this paucity of data prompted us to undertake the present evaluation. This study was undertaken as an attempt to understand the role of pulmonary aspergillosis as opportunistic mycoses in HIV/AIDS patients with pneumonic involvement, and to explore the diagnostic utility of serum galactomannan assay as an adjunct to microscopy and culture for the diagnosis of invasive pulmonary aspergillosis.

METHODS

The present prospective, cross-sectional study was conducted at a tertiary care health facility in North India. The study population comprised 71 HIV-positive subjects presenting to the antiretroviral center or admitted in the ward with signs and symptoms suggestive of a lower respiratory tract infection. The participants of this cohort were recruited during a 3-year period from October 2008 to September 2011.

At the time of enrollment, a detailed history and clinical examination were performed in all patients, the details of which were noted in a pre-designed proforma. A special attempt was made to explore the risk behavior of subjects and to elucidate the possible modes of HIV transmission. After a complete physical examination, routine laboratory investigations, CD4 counts, and chest radiographs were obtained. Written informed consent was obtained from each patient before enrollment, and the study had the approval and ethical clearance from the institutional review board.

Sputum samples were collected in sterile wide-mouthed screw capped containers and immediately transported to the laboratory for processing. An aliquot of each sputum sample was transferred to an Eppendorf containing 10% potassium hydroxide (KOH) and incubated overnight at 37°C. This was primarily done to aid in the dissolution of cellular debris. Subsequently, a wet mount was prepared and examined under ×40 objective of the microscope for any fungal elements.

In addition, the specimens were inoculated on Sabouraud dextrose agar. For each sputum sample, cultures were put up in duplicate and incubated both at 25° and at 37°C. The cultures were examined every second day for up to 6 weeks before they were reported as sterile. Any suspected fungal growth during this period was examined for gross colony morphology including color, texture, topography and the underside or reverse. Further confirmation of the isolates was done by slide culture technique, preparing lactophenol cotton blue mount and examining with the aid of ×10 and ×40 objective of the microscope. Identification of *Aspergillus* species was done based on their characteristic morphological features and employing tests as per standard protocol.[16] Any positive result on microscopy and/or culture was confirmed by subsequent sampling and repeat demonstration/isolation.

Apart from the sputum sample, 2-5 ml blood sample was collected from each case in a sterile centrifugation tube by venepuncture following universal precautions. Following clot retraction, serum was separated by centrifugation and stored in a labeled Eppendorf. All sera were preserved at −70°C till tested for galactomannan antigen. Galactomannan testing in serum samples was done employing the commercially available immunoenzymatic sandwich microplate assay, the Platelia™ Aspergillus EIA (BioRad laboratories). With each run of samples appropriate negative control, cutoff control and positive control sera were included in the study. After the validity of the test run was verified, the presence or absence of galactomannan antigen in the test sample was determined by calculating an index for each patient specimen, which was defined as a ratio of the optical density (OD) value of the specimen to the mean OD of the wells containing cutoff control serum. All patient sera with an index ≥0.50 were considered positive for galactomannan antigen. Data entry was performed using Microsoft Excel sheet and all the entries double-checked for any possible keyboard errors. Data were analyzed using the Epi info software, version 3.5.3, CDC, Atlanta, GA, USA. Descriptive statistics were calculated with arithmetic mean and standard deviation for central tendencies and median for non-normal/skewed distributions.

RESULTS

The age of the study population ranged from 17 to 61 years with a mean age of 33.01 ± 9.15 years. The study group comprised 52 (73.2%) males, 16 (22.6%) females, and 3 (4.2%)

transgenders. 57 (80.3%) of the 71 participants were married while 14 (19.7%) were unmarried. The commonly reported modes of HIV transmission were sexual in 61 (85.9%), intravenous drug abuse in 11 (15.5%), and blood transfusion in 4 (5.6%) of the total 71 study subjects. The chief presenting complaints were fever (49 of 71; 69%), cough (43 of 71; 60.6%), and dyspnea (27 of 71; 38%). While 50 (70.4%) of the study participants were ambulatory and recruited from the antiretroviral center, 21 (29.6%) were hospitalized at the time of enrolment. 42 (59.2%) participants had active pulmonary tuberculosis and 14 (19.7%) had a history of prior pneumocystis pneumonia or had present active disease. None of the patients was neutropenic. The main roentgenographic features noted were a normal chest radiograph in 37 (52.1%), pleural effusion in 22 (31%), infiltrates in 6 (8.5%), opacity in 3 (4.2%), consolidation in 1 (1.4%), hyperinflation in 1 (1.4%), and pneumothorax in 1 (1.4%). The mean and median CD4 count of the study population was 194.70 ± 116.79 and 178 cells/µl, respectively, with a range of 16-445 cells/µl. A summary of the sociodemographic and CD4 profile of the study group is given in Table 1.

Aspergillus species were recovered from 12 (16.9%) sputum samples. Direct microscopy for fungal hyphae was positive in 7 (9.9%) samples (all positive for *Aspergillus* species on culture). The Platelia™ Aspergillus EIA test for serum galactomannan antigen was positive in 36 (50.7%) patients. A summary of the modalities employed and their diagnostic yield is given in Table 2.

Employing a positive microscopy (dichotomously branched septate hyphae seen in KOH wet mount) and culture as a diagnostic criterion, pulmonary aspergillosis was diagnosed in 7 (9.9%) patients, 5 of whom showed a positive antigenemia and were thus considered to be suffering from invasive forms of pulmonary aspergillosis.

The prevalence of pulmonary aspergillosis was highest in individuals 21-40 years of age (13.3%), and none of the cases occurred in individuals above 41 years of age. The mean age of the patients with pulmonary aspergillosis was 29.57 ± 6.02 years. Among the patients with pulmonary aspergillosis (7); 4 (57.1%) were males and 3 (42.9%) were females. The prevalence of pulmonary aspergillosis was more in females (18.7%; 3 of 16) as compared to males (7.7%; 4 of 52). 2 (28.6%) of the 7 patients with pulmonary aspergillosis had active pulmonary kochs; 2 (28.6%) had concomitant pneumocystis pneumonia and one (14.3%) had dual infection (both tuberculosis and pneumocystis pneumonia). While 3 (42.8%) of these patients had a normal chest radiograph on presentation, 2 (28.6%) presented with infiltrates and 2 (28.6%) with pleural effusion. 2 (28.6%) of the seven patients with pulmonary aspergillosis had a CD4 count less than 100 cells/µl. The mean CD4 count of the patients with pulmonary aspergillosis was 155.86 ± 119.33 cells/µl (median = 117 cells/µl; range = 18-329 cells/µl). A comparison of the CD4 counts of patients with and without pulmonary aspergillosis is given in Table 3.

Table 1: Sociodemographic and CD4 profile of the study group (*n*=71)

Variable	Number (%)
Age groups in years	
≤20	7 (9.9)
21-30	28 (39.4)
31-40	23 (32.4)
41-50	10 (14.1)
≥51	3 (4.2)
Sex	
Male	52 (73.2)
Female	16 (22.6)
Transgender	3 (4.2)
Marital status	
Married	57 (80.3)
Unmarried	14 (19.7)
Residence	
Rural	15 (21.1)
Urban	56 (78.9)
HIV status of partner	
Positive	30 (42.3)
Negative	15 (21.1)
Not tested	26 (36.6)
CD4 count	
<100	18 (25.4)
100-200	20 (28.2)
201-350	26 (36.6)
>350	7 (9.8)

HIV: Human immunodeficiency virus

Table 2: Diagnostic yield of various methods

Diagnostic technique	Number of samples positive (%)
KOH mount alone	0 (0)
Culture alone	0 (0)
Serum galactomannan alone	26 (36.6)
KOH mount+culture alone	2 (2.8)
KOH mount+serum galactomannan alone	0 (0)
Culture+serum galactomannan alone	5 (7)
KOH mount+culture+serum galactomannan	5 (7)

KOH: Potassium hydroxide

Table 3: A comparison of CD4 profile of HIV positive patients with and without pulmonary aspergillosis

Study group	Mean	Median	Range
Patients with pulmonary aspergillosis (*n*=7)	155.86±119.33	117	18-329
Patients without pulmonary aspergillosis (*n*=64)	198.95±116.68	190	16-445

HIV: Human immunodeficiency virus

The common *Aspergillus* species isolated from the sputum samples of these patients were *Aspergillus flavus* (4; 57.1%), *Aspergillus fumigatus* (2; 28.6%), and *Aspergillus niger* (1; 14.3%). A line listing of the patients with pulmonary aspergillosis along with their demographic, clinical and microbiological profile is summarized in Table 4.

DISCUSSION

Alhough *Aspergillus* species were initially considered uncommon pathogens in HIV disease, several series of patients with advanced HIV disease and *Aspergillus* respiratory infections, particularly the invasive forms, have been reported.[3,9,10,17] Employing a positive direct microscopy, culture and/or serum galactomannan antigen as a diagnostic criterion, 7 cases of pulmonary aspergillosis, including 5 cases of invasive disease were identified in this study cohort of HIV-positive patients. In another study conducted by the present authors to describe the clinicomycological profile of HIV-positive patients with suspected fungal infections, 4 of 60 patients were diagnosed as probable cases of invasive aspergillosis (positive antigenemia), while 1 patient, was diagnosed as a proven case of invasive pulmonary aspergillosis (positive direct microscopy, culture, and antigen detection).[18] On the other hand in a study population comprising 160 confirmed HIV-positive patients with a lower respiratory tract infection, Khan *et al.* diagnosed pulmonary aspergillosis in only 4.[19] The varying prevalences in different studies can be explained by the different inclusion criteria employed, differences in the study population, the diagnostic modalities employed, and the defining criteria used.

While the prevalence of pulmonary aspergillosis was highest in individuals 21-40 years of age, it dropped dramatically among patients above 40 years. A plausible hypothesis is the influence of age and hormonal factors in aspergillosis.[20] Moreover, the high-risk behavior of patients in this age category that predisposes them to HIV/AIDS is an important influencing factor.

Evidence from previous studies suggests that fungal pathogens such as *Aspergillus* species be considered a possible differential diagnosis in HIV-positive patients with CD4 counts below 100 cells/μl.[21] While some studies have attributed the increased prevalence of pulmonary aspergillosis in this group to neutrophil depletion seen in advanced stages of HIV infection, we did not detect neutropenia in any of our patients with a positive microbiological diagnosis of pulmonary aspergillosis.[8] The increased prevalence of aspergillosis in these patients can be explained by the various qualitative or functional abnormalities seen in neutrophils and macrophage lineages in HIV-positive patients with very low CD4 counts.[22] Impaired neutrophil oxidative burst mechanisms in these patients have been previously reported.[22] A study conducted among HIV-infected children found that in study participants with low CD4 counts the antifungal activity of neutrophils against *Aspergillus* hyphae was impaired *in vitro*.[23]

Five of the 7 patients with pulmonary aspergillosis in our study had a positive history of tuberculosis and/or *Pneumocystis carinii* pneumonia. An association between HIV-associated aspergillosis and a history of other AIDS-defining opportunistic infections has also been noted in several previous reports.[24] While *P. carinii* pneumonia and its complications damage the lung architecture and provide a favorable niche for the colonization and growth of *Aspergillus* species, the high dose steroid therapy for its treatment may further deteriorate the immune status of the patient and make them more susceptible to this fungal infection.[24-26] Similarly, tuberculosis of the lung also often leaves behind a scarred pulmonary parenchyma vulnerable to fungal colonization.

Thus, the reasons for development of pulmonary aspergillosis in HIV-positive patients are several and include advent of highly active antiretroviral therapy that has prolonged the survival of patients in advanced stages of AIDS, i.e., with very low CD4 counts, qualitative neutrophil and macrophage dysfunction, prior or co-existing opportunistic lung infections such as tuberculosis and *P. carinii* pneumonia and increased use of corticosteroids and broad-spectrum antibiotics for the prophylaxis and treatment of opportunistic infections.[3,9,10,22-28] Since the sample size of the study group as well as the number of cases that were eventually diagnosed with pulmonary aspergillosis was small, meaningful statistical correlation with any clinical sign and symptom, risk factors and CD4 profile could not be established.

Table 4: Case description of HIV-positive patients with pulmonary aspergillosis (*n*=7)

Age	Sex	Clinical presentation	Mode of HIV transmission	Underlying lung disese	CD4 count	Chest radiograph findings	Direct microscopy	Fungal culture	Aspergillus species isolated	Aspergillus antigen
34	M	Fever, cough	Heterosexual contact	PCP	120	NAD	Fungal hyphae seen	Positive	*Aspergillus fumigatus*	Positive
37	F	Fever, cough	Heterosexual contact	PCP	117	NAD	Fungal hyphae seen	Positive	*Aspergillus fumigatus*	Positive
32	M	Fever, cough	Heterosexual contact	-	83	Infiltrates	Fungal hyphae seen	Positive	*Aspergillus niger*	Positive
18	M	Fever, cough	Heterosexual contact	TB	317	Pleural effusion	Fungal hyphae seen	Positive	*Aspergillus flavus*	Positive
29	F	Fever, cough	Heterosexual contact	-	329	NAD	Fungal hyphae seen	Positive	*Aspergillus flavus*	Positive
28	M	Fever, cough	Heterosexual contact	TB	107	Pleural effusion	Fungal hyphae seen	Positive	*Aspergillus flavus*	Negative
29	F	Fever	Heterosexual contact	TB, PCP	18	Infiltrates	Fungal hyphae seen	Positive	*Aspergillus flavus*	Negative

M: Male, F: Female, HIV: Human immunodeficiency virus, PCP: *Pneumocystis carinii* pneumonia, TB: Tuberculosis, NAD: No abnormality detected

With regard to the diagnosis of pulmonary aspergillosis, we found that the yield was highest with Platelia™ Aspergillus EIA test for serum galactomannan antigen. The test was positive in 26 patients with respiratory disease but without any culture and/or direct microscopic evidence of *Aspergillus* species in sputum samples. While some of these cases could correspond to real circulation of *Aspergillus* antigens implying that the test picked up more positive samples than the conventional methods, the possibility of false-positive results can also not be ruled out. A false positive rate of up to 14% has been reported in a study.[29] The reasons for false positive results are many and include consumption of milk preparations containing high concentrations of galactomannan, patients receiving piperacillin/tazobactam or some batches of amoxicillin/clavulanic acid parenteral preparations, patients on dialysis, those with chronic graft-versus-host disease and cross-reactivity against *Penicillium* species*, Alternaria, Paecilomyces, Fusarium*.[13,30-35] The specificity of antigen detection can be increased by serial sampling. False positivity rates were shown to decline from 54.2% to 11.2% if positivity for at least two samples was taken as a defining criterion.[36] However, since all the positive results in our study were confirmed by subsequent repeat sampling and testing, false positive results seem to be less likely and it is probably the low sensitivity of the traditional methods (microscopy and culture), that a number of positive cases were missed.

Our study highlights that *Aspergillus* species account for a significant proportion in the etiology of lower respiratory tract infection in HIV-positive patients. The relative lack of any pathognomic sign and symptoms and the need for early diagnosis keeping in view the poor prognosis associated with this condition calls for a combination of modalities (microbiological, histopathological, and serological) to make a prompt diagnosis and institute appropriate and effective antifungal therapy. Conventional diagnostic modalities including culture and histopathology are fraught with limitations. Mere isolation of *Aspergillus* species from respiratory specimens does not differentiate colonization from infection, making it imperative to document tissue invasion to attribute any clinical significance to the isolate. However, the serious underlying condition of the patient often does not permit invasive diagnostic interventions such as biopsy for this purpose. In addition, the sensitivity of microscopy and culture is also generally low. These shortcomings are overcome by the Platelia™ Aspergillus EIA assay, which in addition to its high diagnostic yield offers several other advantages. Serum galactomannan can be detected at an early stage of infection, usually, several days before the clinical signs and chest radiographic abnormalities become evident and before a positive culture can be obtained and thus allows for an earlier diagnosis.[37,38] Moreover, the test is specific, non-invasive and, can be repeated at frequent intervals and without removing patients from protective isolation.

The study has a few limitations. First, keeping in view the serious underlying condition of the patients and for ethical reasons, confirmation of the results by tissue diagnosis could not be accomplished, therefore, false results could not be identified, and the true diagnostic performance of the Platelia™ Aspergillus EIA could not be estimated. The second limitation was the relatively small sample size that precluded any meaningful statistical analysis.

CONCLUSION

Our study highlights that pulmonary aspergillosis should be considered a possible differential of pulmonary infection in HIV-positive patients with low CD4 counts. The dismal prognosis associated with the disease calls for early diagnosis and aggressive antifungal therapy. The diagnostic dilemma encountered with the currently used modalities prompts their use in combination and also necessitates the need to include screening for serum galactomannan antigen as a possible diagnostic tool.

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