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Detection of *Aspergillus* Galactomannan in Blood for Early Diagnosis of Pulmonary Aspergillosis in HIV Positive Subjects with and without Tuberculosis in Calabar, Nigeria

Doreen Mmaette Michael Nyong[1], Ofonime Mark Ogba[1*], Oluwayemisi Agnes Olorode[2], Nseobong Godwin Akpan[3], Glory Philemon Bebia[1]

[1]*Department of Medical Bacteriology, Virology and Mycology, Faculty of Medical Laboratory Science, University of Calabar, Nigeria*
[2]*Department of Medical Laboratory Science, Faculty of Health Sciences, Niger Delta University, Bayelsa State*
[3]*Institute of Biomedical Research and Innovations, College of Health Sciences, University of Uyo, Nigeria*

Abstract

This study was aimed at determining the prevalence of pulmonary aspergillosis using *Aspergillus* galactomannan detection in human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) subjects with and without tuberculosis. The study was a prospective cohort study carried out at the University of Calabar Teaching Hospital and Dr. Lawrence Henshaw Memorial Hospital in Calabar. Subjects were HIV-positive patients with and without tuberculosis. Blood and sputum samples were obtained from 215 subjects who consented to the study. A structured questionnaire was administered to assess demographic data and medical history. Sputum samples were subjected to direct microscopy, culture, and Ziehl-Nelseen test. *Aspergillus* galactomannan assay and CD4 counts were performed on the blood samples. The results of *Aspergillus* galactomannan-positive subjects were communicated to the attending Physicians for proper patient management. *Aspergillus* species were recovered from 35(16.3%) sputum samples. *Aspergillus fumigatus* was the most encountered isolate 18(8.4%). The most common presenting symptoms were cough 35(100%) and fatigue 30(85.7%). Out of the 35 subjects with *Aspergillus* isolates, 21(60.0%) had TB, 9(25.7%) had no TB and 5(14.3%) had co-morbidity of HIV and TB. All the subjects with aspergillosis had CD4 counts less than 200 cellsμl^{-1} and there was a statistically significant association between pulmonary aspergillosis and CD4 levels (H=7.02; p = 0.03). Galactomannan assay could be used for early diagnosis of invasive aspergillosis and timely management of patients as it is sensitive, cost-effective, and has a turnaround time of less than 1 hour compared to culture methods.

Keywords: Pulmonary*, Aspergillus* galactomannan, culture, HIV/AIDS, tuberculosis

Резюме

Това проучване е насочено към проучване разпространението на белодробната аспергилоза чрез определяне наличието на галактоманан от *Aspergillus* sp. при човешки имунодефицитен вирус/синдром на придобита имунна недостатъчност (ХИВ/СПИН) на здрави субекти и такива с туберкулоза. Проведено е проспективно кохортно проучване в болницата за обучение на Университета в Калабар и мемориалната болница на д-р Лорънс Хеншоу в Калабар. Субектите са ХИВ позитивни пациенти с и без туберкулоза. Взети са проби от кръв и храчка от 215 субекта, които са дали съгласие за изследването. Въведен е структуриран въпросник за оценка на демографските данни и медицинската история. Пробите от храчки са подложени на директна микроскопия и тест на Ziehl-Nelseen. Кръвните проби са анализирани за галактоманан от *Aspergillus* и брой на CD4 клетки. Резултатите от положителни за галактоманан субекти са съобщени на лекуващите лекари за правилно лечение на пациентите. Видовете *Aspergillus* са открити от 35 (16.3%) проби от храчки. *Aspergillus fumigatus* е най-често срещаният изолат 18 (8.4%). Най-често срещаните симптоми са кашлица 35 (100%) и умора 30 (85.7%). От 35 субекта с изолати на *Aspergillus*, 21 (60.0%) са имали туберкулоза, 9 (25.7%) не са имали туберкулоза, докато 5 (14.3%) са имали коморбидност на ХИВ и туберкулоза.

* Corresponding author: ofonimemark@yahoo.com; ofonimemark@unical.edu.ng
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Всички субекти с аспергилоза са имали брой CD4 клетки под 200 μl-1 и има статистически значима връзка между белодробната аспергилоза и нивата на CD4 (H=7,02; p = 0,03). Анализът на галактоманан може да се използва за ранна диагностика на инвазивна аспергилоза и навременно лечение на пациентите, тъй като е чувствителен, рентабилен и има време за изпълнение по-малко от 1 час в сравнение с методите за култивиране.

Introduction

Chronic pulmonary aspergillosis (CPA) affects about 3 million people of which 1.2 million are co-infected with pulmonary tuberculosis. In underdeveloped countries, the burden of CPA is close to that of tuberculosis (Denning, 2017). Invasive aspergillosis is associated with patients with suppressed immune systems as a result of low neutrophils that are responsible for eliminating the fungi in the system. Aspergillosis occurs due to airway or lung invasion, or extrapulmonary dissemination. Apart from an impaired immune status, factors like quantity, duration of exposure to the fungi are important determinants of host response. *Aspergillus* species are linked to numerous diseases in both man and animals and are regarded as opportunistic pathogens (Barton, 2013; Denning *et al.,* 2013).

The most common forms of the disease include; chronic pulmonary aspergillosis, allergic bronchopulmonary aspergillosis, and invasive aspergillosis. Symptoms of aspergillosis depend on the specific form of the disorder present. The condition of the host immune system determines to a large extent the clinical features, course, and severity of the infection (Kosmidis and Denning, 2015). The Centre for Disease Control (CDC) reported that most people inhale *Aspergillus* spores every day without falling ill, but those with lung infections or weak immune systems are more liable to develop health problems associated with *Aspergillus.* Invasion of the tissues is rare and occurs mostly in immunosuppression associated with therapy for haematopoietic cell transplantation, haematological malignancies, or solid organ transplantation. Annually, the chronic, invasive, and allergic forms of aspergillosis together account for about 600,000 deaths globally (Nam *et al.*, 2010; Chen *et al.,* 2013; Kosmidis and Denning, 2015). In the USA, aspergillosis accounts for a hospitalization rate of 36 million persons per year (Tong *et al*., 2009). The analysis of hospital discharge and other medical data shows that invasive aspergillosis is connected with a high level of mortality, length of stay, and hospital costs compared to similar patients without invasive aspergillosis (Barton, 2013).

Despite recent advances in antifungal therapy, some forms of aspergillosis still cause increasing mortality and morbidity, partly due to difficulties and delays experienced in laboratory diagnosis (Powers-Fletcher *et al*., 2016). Early diagnosis and initiation of the correct chemotherapy have been shown to have a positive clinical impact on patients (Verweij *et al*., 2009).

Diagnosing aspergillosis has been problematic because the culture of specimens and microscopy lack sensitivity, on the other hand, tissue biopsy for histopathology is rarely obtainable, and radiographic imaging is not organism-specific. The detection of different *Aspergillus*-specific antibodies and metabolites of *Aspergillus* species are additional underused diagnostic techniques (Hope *et al.,* 2005). Thus, more attention should be given to non-culture-based methods like the detection of 1, 3-β-D-glucan, and galactomannan antigens which are more sensitive and reliable (Pfeiffer *et al*., 2006). This study aimed to determine the prevalence of pulmonary aspergillosis using *Aspergillus galactomannan* detection in human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) subjects with and without tuberculosis.

Materials and Methods

The study was carried out at the University of Calabar Teaching Hospital (UCTH) and Dr. Lawrence Henshaw Memorial Hospital (DLHMH), located in Calabar municipality and Calabar South LGA respectively. The two Hospitals are anti-retroviral therapy (ART) and Directly observed treatment short course (DOTS) centers. This study was a cohort study that ran for twelve months from December 2019 to November 2020. Ethical clearance was obtained from the Health Research and Ethical Committee of the University of Calabar Teaching Hospital with Protocol Assigned number: UCTH/HREC/33/677. Informed consent was obtained from subjects. Those with pulmonary symptoms who were able to produce sputum were enrolled. On the other hand, subjects who could not produce sputum and those who had taken antifungal treatment in the past 2 months were excluded from the study. A pre-designed questionnaire was administered for data on demography and medical history.

Sample collection and processing

Two hundred and fifteen subjects were enrolled for the study. Venepuncture was performed

on subjects and 5mls of blood was obtained for CD4 count determination and *Aspergillus* galactomannan detection. Sputum samples were obtained twice from patients with productive cough in sterile wide-mouthed screw-capped containers and transported to the Laboratory for analysis. The sputum analysis was carried out under level 2 Biosafety cabinets. Sputum samples were subjected to macroscopy, microscopy, and fungal culture. The sputum sample was mixed in an equal volume of 0.5% pancreatin and centrifuged for 10 minutes at 3000 rpm. The sediment was vortexed and used for fungal culture and direct microscopic examination in 20% KOH solution (Cheesbrough, 2006). The sputum samples were inoculated unto Sabouraud dextrose agar supplemented with chloramphenicol (50µg/ml) and incubated at 30°C. Cultures were examined twice weekly for 4 weeks. Wet mounts using KOH were performed to check for the presence of fungal elements. Zeihl-Neelsen staining was carried out to determine the TB status of subjects (Oladele *et al.,* 2017).

Aspergillus galactomannan assay

The *Aspergillus* galactomannan assay was carried out with the lateral flow test kits obtained from (IMMY, Alpha Laboratories, United Kingdom). Two mL of serum was used for the galactomannan test. A micropipette was used to dispense 300 µL of serum into the first centrifuge tube. Pre-treatment buffer (100 µL) was added to the sample in Tube 1 and vortexed. The sample was placed on a heat block for 8 minutes at 120°C and spun at 12,000 g for 5 minutes. About 80 µL of the spunned sample was transferred from Tube 1 to Tube 2. Then 40 µL of *Aspergillus* galactomannan running buffer was added to Tube 2. The test strip was inserted (↓↓ down) and allowed for 30 minutes before readings were taken (Barton, 2013; Oladele *et al*., 2017). Two colour bands indicated positive results while singe colour band on the control line indicated negative results.

Identification of isolates

Significant growths were identified on the basis of gross morphology, topography, pigmentation, and microscopic appearance in lactophenol cotton blue mount viewed with an x40 objective lens. Any positive result on microscopy and/or culture was confirmed by repeated demonstration of isolates from multiple samples.

Data analysis

The data analysis was performed using Statistical Package for Social Sciences (SPSS) version 20.0 software (IBM Corp, Armonk, NY, USA). Descriptive statistics was for categorical variables. Quantitative and qualitative variables were expressed as absolute values and percentages. Dependence with each variable was analyzed using the chi-square test. The p-value of ≤ 0.05 was considered statistically significant.

Results

A total of 215 subjects were recruited for the study. Subjects were aged 16 to 75 years with a mean age of 37.9±14.5 years. The highest number of subjects were aged 16 to 25 years 55 (25.6%). Subjects were more of the male gender 123 (57.2%). There was an almost equal number of married subjects 109 (50.7%) to single subjects 106 (49.3%). Most of the subjects were residents of Calabar South 145 (67.4%). The most common underlying illness among the study population was tuberculosis 136 (63.3%) followed by HIV 49 (22.8%). There was HIV/TB co-morbidity among 30 (13.9%) subjects (Table 1).

Table 1. Socio-demographic characteristics and underlying illnesses of subjects

Variable	No. (%) of subjects (n=215)
Age (years)	
16-25	55 (25.6)
26-35	48 (22.3)
36-45	54 (25.1)
46-55	31 (14.4)
56-65	22 (10.2)
66-75	5 (2.3)
Gender	
Male	123 (57.2)
Female	92 (42.8)
Marital status	
Single	106 (49.3)
Married	109 (50.7)
Underlying illness	
Tuberculosis	136 (63.3)
HIV	49 (22.8)
HIV and Tuberculosis	30 (13.9)

Out of the 35 subjects with culture-positive *Aspergillus* infection, those aged 26-35 years had the highest infection rate (22.9%) while subjects aged 66 years and above had the lowest infection rate 1 (20.0%). There was no statistically significant relationship between the age of subjects and infection rates (χ^2=4.45; p = 0.6) (Table 2).

Table 2. Distribution of *Aspergillus* infection by age of subjects

Age	No. of subjects examined	No. (%) with *Aspergillus* infection	Statistics
16-25	55	7 (12.7)	$\chi^2 = 4.45$
26-35	48	11 (22.9)	P = 0.60
36-45	54	7 (13.0)	
46-55	31	4 (12.9)	
56-65	22	5 (22.7)	
66 and above	5	1 (20.0)	
Total	215	35(16.3)	

The male gender had a higher *Aspergillus* infection rates 23/123 (18.7%) than females of 12/92 (13.0%) (Fig. 1).

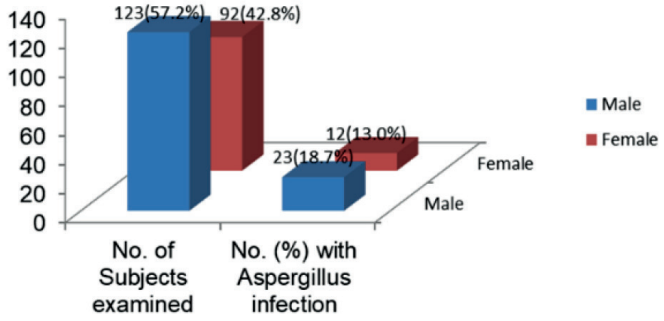

Fig. 1. Distribution of *Aspergillus* infection by gender of subjects

Out of the 35 isolates of *Aspergillus* species encountered in the study, *Aspergillus fumigatus* was the most prevalent isolates 18(51.4%) followed by *A. niger* 9(25.7%) while *A. flavus* was the lowest encountered isolate 8(22.9%) (Fig. 2).

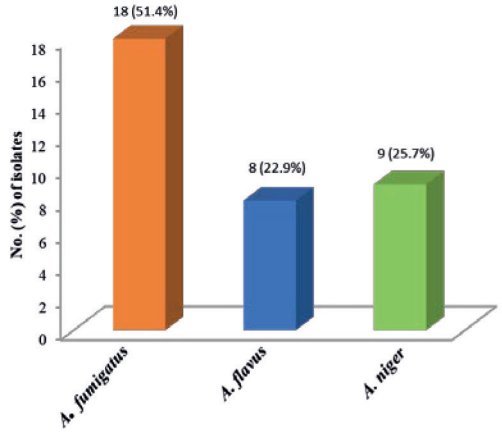

Fig. 2. Distribution of *Aspergillus* isolates among subjects

Subjects with *Aspergillus* infection had a mean CD4 count of 170.2±57.72. Those with *A. fumigatus* infection had a mean CD4 count of 189.8±65.2 while those with *A. niger* and *A. flavus* infections had mean CD4 counts of 125.6±40.8 and 170.2±30.7 respectively. There was a statistically significant effect of the immune status of subjects on *Aspergillus* infection rates (Kruskal-Walis H = 7.02, p = 0.03) (Table 3).

In this study, the highest rate of aspergillosis 21(60.0%) occurred among TB patients followed by HIV patients 9(25.5%) while 5(14.3%) infection rates occurred in HIV-TB co-morbidity (Fig. 3).

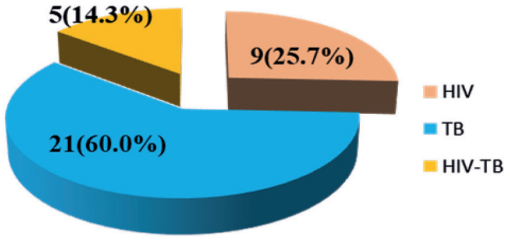

Fig. 3. Distribution of Aspergillosis in association with other infections

A total of 100 subjects were tested for *Aspergillus* galactomannan antigens. This included the 35 microscopy/culture-positive cases. Out of the 100 subjects, 26(26.0%) were positive for the galactomannan antigens (Table 4).

Discussion

Aspergillosis is a life-threatening fungal infection in immunocompromised patients, including people infected with Human immunodeficiency virus (HIV) and those having lung

Table 3. Distribution of *Aspergillus* species by mean CD4 counts of subjects

Aspergillus species	No. (%) with *Aspergillus* infection	Mean CD4 counts (cells μl^{-1})	Statistics
A. fumigatus	18 (51.4)	189.8±65.2	Kruskal-Walis H=7.02
A. flavus	8 (22.9)	125.6±40.8	P = 0.03
A. niger	9 (25.7)	170.2±30.7	
Total	35(16.3)	170.257.72	

Table 4. Diagnostic yield of *Aspergillus* galactomannan LFA and cultural technique

Diagnostic method	No (%) positive
Aspergillus galactomannan (n = 100)	26(26.0)
Culture (n = 215)	35/215(16.3)

diseases like Tuberculosis (TB). Low immunity level predisposes these patients to pulmonary fungal infections. In developing countries like Nigeria, *Aspergillus* infections are gradually becoming major health challenges as there are limitations to early and effective diagnosis of this disease (Powers-Fletcher *et al.,* 2016).

In this study, *Aspergillus* species were recovered from 16.3% of sputum samples via cultural characteristics. This is consistent with a study in Maiduguri, done by Talle *et al.* (2017) with a prevalence of 18.6%. The works reported by Ogba *et al.*(2016) in Calabar, Nigeria (6.3%) and Kaur *et al.* (2017) in India (9.9%) showed a lower prevalence respectively when compared to the present study. The high prevalence rates recorded in this study could be due to the inclusion of study subjects with pulmonary tuberculosis (TB), most of whom had not commenced the treatment process.

Of the 35 cases of aspergillosis, a total of 26 subjects had TB. Tuberculosis has been shown to be a high-risk factor for chronic pulmonary aspergillosis (CPA). Nigeria is among the first 5 countries to have a high burden of TB in the world and the second in Africa, with an estimated mortality rate of 81 cases per 100,000 population (Denning *et al*., 2013). The presence of cavities in the lungs as a result of pulmonary TB makes it easy for *Aspergillus* conidia to invade, colonize, and cause infection.

The reasons for the development of pulmonary aspergillosis in HIV-positive patients include the use of highly active antiretroviral therapy (HAART) that prolongs the survival of patients in advanced stages of AIDs having neutrophils and macrophage dysfunction with low CD4 counts. An increase in the use of broad-spectrum antibiotics and corticosteroids also precipitate these effects (Kaur *et al*., 2017). However, this study did not investigate the use of these drugs among subjects.

Several studies have shown that *A. fumigatus* predominantly colonizes and infects the respiratory tract in different clinical conditions including TB. *A. fumigatus* was the most predominant species 18(51.4%) in this study. Ogba *et al.*(2013) in Calabar and Hedayati *et al.*(2015) in Iran also reported *A. fumigatus* as the most prevalent species among their subjects. On the other hand, Amiri *et al.*(2016), reported the most prevalent *Aspergillus* isolates as *A. flavus* in TB patients in Iran. The variation may be due to differences in geographical location.

The highest *Aspergillus* infection (31.4%) occurred in the age group 26-35 years. These findings correlate with the report of Khan *et al.* (2013) in India. The high rate of infection in the 2nd and 3rd decade of life may be due to high HIV infection in this age group, involvement in smoking, and substance abuse. However, age was found not to be significantly associated with aspergillosis infection rates (P = 0.60).

In this study, pulmonary aspergillosis occurred more in males (65.7%) than their female counterparts (34.3%). Our report is similar to the study by Talle *et al.* (2017) who reported 69.3% infection rates in males compared to 66.1% in females. This may be due to a higher level of outdoor activities among male subjects with higher infectivity among artisans and farmers. Included among the artisans in our study were construction workers. This vocation and other earth-moving works have been indicated as predisposing factors to *Aspergillus* infection where the subjects are exposed to infective conidia of *Aspergillus* species via inhalation.

In this study, the mean CD4 count for subjects with pulmonary aspergillosis was 170.2±57.7. Cases of aspergillosis were predominant in patients with CD4 counts less than 200 cellsμl^{-1}. The CD4 cells were found to be significantly associated with aspergillosis (H = 7.02; p = 0.03).

Out of the 35 positive cases for pulmonary aspergillosis, 26 (26.0%) were galactomannan positive. This revealed that (26.0%) of the pulmonary aspergillosis cases were invasive forms as *Aspergillus* galactomannan lateral flow assay can only detect the invasive forms of aspergillosis. Khan *et al.* (2013) reported that invasive pulmonary aspergillosis (IPA) should be considered when patients with late-stage HIV infection or those who have had infections related to HIV such as TB, have CD4 counts <100cellsμl^{-1}. Thus, it could be inferred that the 26 subjects out of the 35 positive cases using *Aspergillus* galactomannan Lateral flow assay and culture were cases of IPA as their CD4 count was lower than 100 cells.μl^{-1}.

According to the European Organization for Research and Treatment of Cancer/Invasive Fungal Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC-MSG), proven invasive fungal infection requires a detection by histological analy-

sis or specimen culture while probable and possible invasive fungal infections require a host factor that identifies the patients at risk, clinical signs and symptoms consistent with the disease and mycological evidence including antigen detection. Thus, 26 out of the 35 positive cases of *Aspergillus* galactomannan antigenaemia could be classified as proven cases, as they were positive for at least 2 diagnostic techniques, had the risk factors, and were immunosuppressed.

Conclusion

This study revealed that galactomannan assay could be used as a timely routine screening for invasive aspergillosis in tuberculosis and advanced HIV for early detection and timely management of patients. Galactomannan assay is cost-effective, has a turnaround time of less than one hour, does not require much labour, serum samples are easily obtained. This study has also revealed a high suspicion index for invasive pulmonary aspergillosis in subjects with tuberculosis and HIV having a CD4 count of less than 100 cells/µl.

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