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Fungal Isolation in HIV Patients and CD4 Count

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Authors' contributions

Author OMTBO designed and supervised the research. Author IBE co-supervised the research. Author AN carried out the research and performed the statistical analysis. Author JOO wrote the protocol, the manuscript and managed the literature searches. Authors COO, MN, IN and CMO managed the analyses of the study and the questionnaire. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: This study aimed to investigate fungal isolation in HIV infected patients and its relationship with CD4 count.
Study Design: Cross-sectional study.
Place and Duration of Study: This study was carried out in Nnamdi Azikiwe University Teaching Hospital, Nnewi, Anambra State, Nigeria (between March and July 2013).
Methodology: A total of 100 positive Human Immunodeficiency Virus (HIV) patients (28 males, 72 females; age range 1-70 years) were included in this study. The sputum specimens were tested for *mycobacteria* using Ziehl Neelson's staining technique. Fungal sputum culture was carried out using standard conventional fungal culture method. Identification was done using chromogenic media and standard staining methods.
Results: There were significant fungal associations with gender, age and antiretroviral therapy ($P \leq 0.05$). Out of 100 sputum samples cultured, 80 had fungal growths; 61 single and19 mixed isolates, while the remaining 20 samples were without fungal growth. Different fungi species were isolated from 5 out of the 9 patients positive for

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Mycobacterium spp. A total of 8 different fungal species were isolated with *Candida albicans, 24(30%),* as the predominant species which had a CD_4 count range of 10-200 cells/µl, while *Aspergillus niveus* was the least, 1(1.2%) with CD_4 count range of 300-400 cells/µl. *Penicillium marneiffei* was the second most prevalent fungi, 11(13.8%). Patients with CD_4 T-cell count of less than 100 cells/µl had the highest frequency of fungal isolates from sputum 27(76.4 %) ($P \leq 0.05$), while those with CD_4 counts >400 cells/µl showed no fungal infection. Patients with *Aspergillus fumigatus*, *Candida glabrata* and mixed infections had a total white blood cell (WBC) count of $<4.0x10^9$ cells /1. Neutropenia was also observed in patients with *Candida albicans, Aspergillus fumigatus, Aspergillus niger and Pencillium marneiffei.*

Conclusion: HIV infection increases the susceptibility to fungal colonization and infection. The CD4 counts of the patients have a strong relationship with the frequency and type of fungal isolates. The lower the CD4 count the higher the frequency of fungal isolates. Since invasive fungal colonization of the lungs remain important causes of death in immunocompromised patients, early isolation and identification of the colonizing fungi can improve the prognosis of patients.

Keywords: Fungal isolation; human immunodeficiency virus; Mycobacterium spp; CD_4 count; white blood cells; antiretroviral therapy.

1. INTRODUCTION

The Human Immunodeficiency Virus (HIV) is a retrovirus that causes AIDS (Acquired Immune Deficiency Syndrome). The retrovirus primarily attacks the immune defense system, making the body extremely vulnerable to opportunistic infections. Colonization and transient superficial fungal infections predispose immunocompromised patients to fungal opportunistic infections. According to the reports of United States Embassy in Nigeria, Nigeria had a national HIV prevalence of 4.1% in 2010 which vary among some groups: children 11%, men 37% and women 57% [1]. Opportunistic infections occur in individuals who have weakened immune systems [2]. HIV can infect and kill many different types of cells in the body but its primary targets are the CD_4 T-cells, which are white blood cells that helps coordinate the immune system's response to infections and diseases [3]. Patients progress to AIDS (acquired immune deficiency syndrome) when their CD4 cell counts drop below 200 cells per micro-liter of blood [4]. Fungi classified as a kingdom of microorganisms encompasses such organisms as yeast and molds. According to Center for Disease Control (CDC) [5], fungal species can be found in normal flora (*Candida albicans* in mouth and gastrointestinal tract), however they can also induce mild and local infections (tinea versicolor, tinea cruris, oral thrush, allergic bronchopulmonary aspergillosis), or invasive opportunistic infections (esophageal candidiasis, cryptococcal meningitis, penicilliosis) in HIV infected or AIDS patients. Most people have been exposed to the disease–causing fungi because they are ubiquitous. However, infections only occur in individuals who have weakened immune systems [3].

Acquired immunodeficiency syndrome (AIDS) caused by HIV Virus has been the precipitating cause for the increased incidence of most common fungal disease such as candidiasis, cryptococcosis, aspergillosis and others. Usually HIV positive individuals also have other predisposing factors such as neutropenia, lymphopenia, frequent use of antibiotics for prophylaxis and treatment of various bacterial infections [6]. The importance of fungal disease among patients with HIV infection was recognized in the early days of the acquired cellular immunodeficiency" in 1981 [7]. The spectrum of the illness ranges from

asymptomatic mucosal candidiasis to overwhelming, disseminated infection and life threatening meningitis caused by *Cryptococcus neoformans* and Histoplasma. Mucosal candidiasis remains the most common fungal infection in this population, while cryptococcus and aspergillosis are associated with significant mortality. Histoplasmosis and penicilliosis are relatively common in some areas. Blastomycosis, coccidioidomycosis and paracoccidiodomycosis have also been described in association with HIV [8]. The risk of fungal colonization and isolation depends primarily on these factors: the severity of impairment of cell-mediated immunity; the risk of exposure; recent or current use of an antifungal medication and neutropenia, which relates primarily to invasive candidiasis and aspergillosis. Impairment of cell-mediated immunity is a predisposing factor to cryptococcosis, histoplasmosis, coccidiodomylosis and mucocutaneous candidiasis [9].

Mycobacterium is a genus of Actinobacteria, given its own family, the genus includes pathogens known to cause serious diseases in mammals, including tuberculosis (*Mycobacterium tuberculosis*) and leprosy (*Mycobacterium leprae*) [10]. Tuberculosis and HIV co-infections pose particular diagnostic and therapeutic challenges. Infection with HIV is the most powerful known predisposing factor to *Mycobacterium tuberculosis* (TB) and *Mycobacterium avium-intracellulare* infection (which can occur at the late stage of AIDS). HIV has been known to be associated with depressed immunity. Previous studies have indicated that TB is the most common opportunistic infection in HIV patients particularly in those with CD_4 counts less than 200 cells/µl [11]. A high prevalence of TB-HIV co-infection, reaching up to 65% of the study population has been reported [12]. Spectrum of Opportunistic Infections (OIs) has been found to vary from continent to continent and region to region [13]. With the unprecedented increase in the number of AIDS cases, OIs are also increasing [13]. Respiratory infections are among the most common causes of morbidity and mortality among human immunodeficiency virus (HIV) patients [14]. Fungi are responsible for various invasive diseases in immunocompromised and cystic fibrosis patients, the most common being allergic bronchopulmonary Aspergillosis. Others include severe asthma, aspergilloma, invasive aspergillosis and chronic necrotizing pulmonary aspergillosis. This study aimed to investigate fungal isolation in HIV infected patients in relation to their CD4 counts.

2. MATERIALS AND METHODS

2.1 Study Location

This study was carried out at Nnamdi Azikiwe University Teaching Hospital (NAUTH), Nnewi, Anambra State, South-Eastern Nigeria, which is a referral centre for both private and public health institutions in and around Nnewi town.

2.2 Ethical Issues

Ethical clearance was sought for and obtained from the ethics committee, Nnamdi Azikiwe University Teaching Hospital. Questionnaires were administered to consenting patients (in the presence of an ethics committee member) so as to obtain both demographic characteristic and health challenges, with an assurance of confidentiality for the information they provided. The reported health challenges observed in the patients included difficulty in breathing, cough, blood in sputum and chest pain.

2.3 Inclusion and Exclusion Criteria

The patients who were in the age range of 1 to 70 years were included in this study. This inclusion was made from HIV positive patients who were from Anambra and its neighboring states (Enugu and Imo) and had cases of oropharyngeal lesions, irritations or respiratory health challenges. The patients were all visiting the centre (NAUTH) for the first time. Patients who were on immunosuppressive drugs, antibiotics and are HIV negative were excluded from the study.

2.4 Sample Size Calculation

The sample size was obtained using the formula stated by Naing et al., [15]; for the calculation of minimum sample size used in a study involving human subjects/patients, which is $n=Z^2xP(1-P)/d^2$. Where

n=minimum sample size
d=desired level of significance (0.05)
Z=confidence interval (1.96)
P=prevalence rate of HIV in Nigeria was 4.1% [1]; (4.1/100=0.041).
$n=1.96^2x0.041\ (1-0.041)/0.05^2$.
n=60 patients

Using the formula, the calculated minimum number of sample size was 60, but a total of 100 participants were included for this study.

2.5 Method of Sample Collection

Three (3) early morning sputum samples were collected for 3 consecutive days from each of the HIV patients. Patients were asked to collect the sample in a sterile wide mouth Stericon container (Evepon, Nigeria) [16]. Five (5) ml of blood sample was collected from each of the patient [16], into sterile plain tubes and EDTA containers for CD_4 cell count and estimation of total and differential white blood cell counts while 2 ml of serum was extracted after centrifugation and used for HIV screening. All samples were aseptically collected and kept between 4°c and -20°C until analyzed.

3. ANALYSIS OF SAMPLE

3.1 Sputum Sample

Cultural Technique: each sample was inoculated on two sets of Sabouraud's dextrose agar (SDA with antibiotic chloromphenicol and SDA only; Lab M, India), incubated at 25°C for two weeks and examined for growth every 3 days. SDA plates with growths were identified macroscopically and microscopically on Agar-block mounts using Lacto-phenol cotton blue stain (Sigma) and Mycology atlas. A criterion for a positive growth was based on two samples from a patient yielding the same growth.

Sputum Samples were examined for Mycobacterium spp. using Ziehl Neelson's staining technique. Positive AFB slides were graded and recorded [16].

3.2 Identification of Fungal Isolates

The different fungal isolates were identified macroscopically and microscopically as described by Mackie and McCartney [17]. Macroscopic identification was based on the colonial morphology of the isolates on the laboratory media/visual examination based on the physical appearance such as: colour, texture, colonial topography and diffusible pigments after Agar–block mount. Mucoid yeast-like growth was processed by carrying out, capsular staining, and inoculation unto ChroMagar candida (France), and confirmed with yeast Identification Kit. (Liofilchem, Spain)Identification was based on the morphology of the spores and hyphae on preparation with lactophenol cotton blue (LCB) wet mount technique. The morphological features of fungal species were observed and identified using Mycology atlas. Gram's staining was done on all the isolates with mucoid yeast-like growth and observed for Gram positive budding yeast cells [16]. All the isolates with mucoid and yeast like growth were tested for capsulated budding yeast cells for identification of *Cryptococcus neoformans* using Indian ink preparation [16]. Christienses' urease test was used to confirm *C. neoforman* [10]. Different Candida species were identified on a selective and differential chromogenic (ChroMagar candida, France). The candida species were identified based on their chromogenic reaction as described in the manufacturer's instruction; *Candida albican* (Green), *C. tropicalis* (Blue), *C. glabrata* (Pink), *C. krusie* (Dry pink).

HIV screening**:** the patients were re-screened for HIV by immuno-chromatographic technique using Determine kit (Alere, Japan) and Stat-pack kit (USA). This was done to rule out false positive results, if any. For CD_4 T-Cell count, Flow cytometry method was used. Total and differential white blood cell count (wbc) was done using an automatic analyzer. (Abbott cell–Dyn 1700).

3.3 Statistical Analysis

Data derived was analyzed using chi-square and correlation co-efficient analysis. Level of significance was set at 95% confidence limit (P-value of 0.05 level of significance). Statistical analysis for social sciences version 17 was used for the evaluation.

4. RESULTS AND DISCUSSION

Table 1 shows the demographical characteristics of the study population in relation to the number of fungal isolates. The number of females (59) infected were found to be significant compared to the number of males (21) infected ($P \leq .05$). The number of patients, 48 (90.5%), who were not on antiretroviral therapy were found to be significantly affected by fungal isolation compared to those patients, 32 (68%), who were on antiretroviral therapy ($P \leq .05$).

Out of 100 sputum samples of HIV patients cultured in this study, a greater number had single fungal isolates than mixed fungal isolation (Table 2). Candida species were isolated in 50.8 % of sputum samples, of which *Candida albicans* was the most frequently isolated fungi (Table 2). Candidiasis has been found to be the most common fungal isolation in HIV/AIDS and is of prognostic value only as its presence indicates progression of immunodeficiency [13]. According to an earlier finding [18], a total of 6 different fungal isolates from sputum sample out of which *C. albicans* predominated was reported. The report also stated that other Candida species (*C. glabrata*) was also isolated. A team of scientists [19] in Punjab isolated *C. albicans* in 20 (62.5%) out of 32 isolates while another

team reported *C. albicans* as the predominate isolate in HIV infected individuals [20]. Other Candida species such as *C. glabrata* 6(7%) were also isolated in this study.

Table 1. Demographic information on the study population in relation to the number of fungal isolates

		No. of pts sampled	No. of pts infected with Fungi	% prevalence	*P*-value	X^2
Sex						
Male		28	21	47.8		
Female		72	59	52.2	.00*	38.10
Total		100	80	100		
Marital Status						
Married	Male	16	14	51.2		
	Female	42	35	48.8		
Total		58	49	100		
Single	Male	12	7	42.2	.22**	0.99
	Female	30	24	57.8		
Total		42	31	100		
Occupation						
Civil servants		8	5	13.5		
Drivers		5	4	17.3		
Farmers		16	14	18.9	.58**	0.78
House wife		12	8	14.4		
Students		30	25	18.0		
Traders		29	24	17.9		
Total		100	80	100		
Antiretroviral Therapy (ART)						
Yes		47	32	42.9		
No		53	48	57.1	.00*	37.77
Total		100	80	100		
Mycobacterium spp. Status						
Positive	Male	2	2	40		
	Female	7	3	60	.01*	15.45
Total		9	5	100		

*P-value is significant at p=.05, n=100, Keys: No: Number, Pt: patients, %: Percentage, *: Significant, **: insignificant, r=correlation co-efficient,x^2=chi-square, n=number of patients included.*

Penicillium marneiffei was the second most common fungi isolated in this study (Table 1). Penicilliosis is the third common opportunistic infection in patients with AIDS [21,22]. It is believed to hasten the emergence of AIDS, though more studies are required. It is always associated with low CD_4 count typically less than 100cells/µl [13]. Single or mixed fungal isolates" of *P. marneiffei* were associated with low CD4 counts (15-200cells/µl) (Table 2). It is very pathogenic and can mimic tuberculosis with 50% cases resulting in cough, dyspnea and hemoptysis. According an earlier study, *Penicillium marneiffei* was isolated from HIV positive individuals whose CD_4 counts were less than 100cells/µl [23].

A total of 8 different species of fungi were isolated singly and in mixed populations of two or more isolates. A total of 61 sputum samples yielded single fungal isolates, 18 yielded 2 isolates while one sample yielded 3 fungal isolates (Table 2).

Table 2. Frequency distribution of different fungal isolates from sputum of HIV patients with their CD4 counts

Fungal isolates	No. isolated	% of Isolate	CD_4 cells/µl
Candida albicans	24	30	10-200
Penicillium marneiffei	11	13.8	50-200
Candida glabrata	7	8.8	50-200
Aspergillus fumigatus	7	8.8	50-200
Aspergillus niger	6	7.5	100-200
Aspergillus flavus	3	3.8	30-100
Cryptococcus neoformas	2	2.5	100-200
Aspergillus niveus	1	1.2	300-400
Total	61		
Mixed fungal isolates			
Candida. albicans and Penicillium marneiffei	11	13.7	50-200
Aspergillus fumigatus and Penicillium marneiffei	4	5.0	10-200
Aspergillus fumigatus and Candida albican	2	2.5	100-200
A. flavus and Candida glabrata	1	1.2	200-300
Aspergillus fumigatus, Candida albican and Penicillium marneiffei	1	1.2	15
Total	19		

X^2: 41.083, P=0.00

The Aspergillus species were isolated in 27.9% of the sputum samples. Out of the four Aspergillus species isolated, *Aspergillus fumigatus* (8.8%) was the most frequently isolated species (Table 2). Aspergillus exposure is universal, though invasive aspergillosis is rare in general, it is commonly found in immunocompromised cases like in HIV infection [14]. In this study, out of 17 samples, 7 cases of *A. fumigatus,* 6 cases of *A. niger,* 3 cases of *A. flavus* and 1 case of *A. niveus* were observed. According to previous report, out of 13 samples, *A. fumigatus* was isolated in 6 samples, *A. niger* was isolated in another 6 samples and *A. flavus* in one sample [18]. Since the findings of this study correlates reasonably well with the previous report stated, *A. fumigatus* and *A. niger* the could be said to be most frequently isolated Aspergillus species in HIV patients.

Cryptococcus neoformans was isolated from 2 samples (2.5%) in this study. This finding is similar to that of a study carried out in Cameroon where *C. neoformans* had an incidence rate of 2.04% in HIV patients [24]. *C. neoformans* was also isolated from 5 samples out of 100 samples of HIV patients in India [25]. This means that its incidence is low and in all cases seems to occur at a low rate. In this study, the CD4 count in the colonized subjects was between 100- 200 cells/µl of the total average cases isolated (Table 2).

The table shows that the age ranges of 21-32 years and 31-40 years had the highest number of positives cases (27 and 24, respectively) for fungal isolates for both males and females. There is a statistical significance ($P \leq 0.05$) in fungal colonization among the age

groups. More so, the age ranges of 31-40 years and 21-32 years had the highest rate of fungal colonization for males and females respectively.

Table 3. Distribution of fungal colonized subjects according to gender (sex) and different age groups

Age range (yrs)	Total no. of male pts sampled	Frequency of infected male pts	Total no. of female pts sampled	Frequency of infected female pts	Total no. of pts sampled
1-10	2	2	1	1	3
11-20	3	3	3	2	6
21-32	6	4	28	23	34
31-40	4	4	24	20	28
41-50	10	7	11	9	21
51-60	3	1	3	2	6
61-70	-	-	2	2	2
Total	28	21	72	59	100

r=0.30, P=.00, P is significant at p=.05, n=100,Keys: Yrs=Years, No=Numbers, Pts=Patients, r=correlation coefficient, n=number of patients included

The correlation between sex (28 males and 72 females) and age (1-70years) on the prevalence of fungal colonization among this group was found to be significant (Tables 3). This shows that fungal colonization is more prevalent in females than males, and more pronounced in some age ranges than the other. This could be related to the fact that females had higher HIV records than the males (Table 3).

More so, the study revealed that there was a statistical significance between the frequency of fungal isolation among those on antiretroviral therapy (ART) and those that are not on ART ($P \leq 0.05$). Out of 47 patients on ART, 32 of them presented cases of fungal colonization while out of 53 patients who were not on ART, 48 of them presented cases of fungal colonization. This indicates that those not on ART are prone to fungal colonization. This could be attributed to the fact that ART reduces the chances of fungal isolation by immune reconstitution with higher CD4 counts.

Table 4. Distribution of fungal isolation in HIV infected patients and its relation with CD_4 Count

CD_4 cell/µl	Frequency of patients sampled	Frequency of infected patients	% prevalence
<100	28	27	96.4
100-200	26	25	96.2
201-300	21	19	90.5
301-400	25	9	36.0
>400	-	-	-
Total	100	80	

X^2: 38.343, P=.01, P–value is significant at p=.05.Keys: µl=Micro litre, %=Percentage, X^2=Chi-square

The table above shows the frequency distribution of fungal isolation in relation to their CD_4 count. Patients with CD_4 count less than 100 cells/µl had the highest frequency of positives isolates, 27(96.4%). The lower the CD4 count, the higher the rate of colonization by fungi.

There was a statistical significance in the result ($P \leq 0.05$). Out of the 100 patients included, 3 were children within the age range of 1-10 years.

The correlation between the CD_4 T Cell count and the frequency of fungal isolation was found to be significant ($P \leq 0.05$) for both single and mixed fungal isolation (Table 4). This implies that the lower the CD_4 Cell counts the higher the prevalence rate. It can be adduced that the depletion of these cells by the virus lowers the CD_4 Count and predisposes these individuals to fungal colonization. This study revealed that all HIV/Mycobacterium spp. cases fall within CD_4 count of <100cells/µl. Patients with CD_4 count of <100cells/µl had the highest frequency of fungal isolates followed by those within the CD_4 range of 100-200cells/µl (Table 4). This finding concurs with an earlier report on HIV infected patients with cases of opportunistic infections [18]. Patients with CD_4 count >400cells/µl showed no fungal isolation.

The table below shows the mean white blood cell total and different count of HIV patients against the different fungal isolates. There was a statistical significance ($P = .05$) in the correlation of the mean total white blood cell count and fungal isolation while no correlation existed between the mean percentage white blood cell differential count ($P > .05$).

Table 5. The total and differential white cell count in relation to fungal isolates

Fungal isolates	No. of isolates	Mean of total WBC count (x 10^9/l)	Mean % Lymphocyte (%)	Mean % Neutrophil (%)
A. flavus	3	4.4	33	67
A. fumigatus	7	3.9	46	54
A. niveus	1	6.0	40	60
A. niger	6	4.4	42	58
C. albican	24	4.2	48	52
C. glabrata	7	3.7	40	60
Cryptococcus neoformas	2	4.9	33	67
P. marneiffei	11	4.4	46	54
A. fumigatus and *P. marneiffei*	4	3.8	45	55
A. fumigatus and *C. albican*	2	3.9	46	54
C. albican and *Penicillium maneiffei*	11	3.7	49	51
Candida glabrata and *A. flavus*	1	4.1	34	66
A. Fumigatus, C. albican and *P. marneiffei*	1	1.5	15	85
r-value		.59	.28	.28
P-value		.04*	.36	.36

*Keys: WBC: White Blood Cell, Spp: Specie, %: Percentage,r: correlation co-efficient *=significant*

In this study, there was a correlation between fungal isolation and the Mean WBC Count (Table 5). This implies that HIV lowers total WBC count and predisposes individuals to fungal colonization and infection. On the other hand, there is no statistical relationship between fungal isolation and differential white cell count (Table 5). Significant correlation may have been observed had it been some histological and radiological investigations were carried out, but for some limitations which include lack of funds and non-patient compliance. From the result, the average neutrophil percentage of patients with Candidiasis and

Aspergillosis were found to be <60% (suggestive of neutropenia). Hence, it could be adduced that neutropenia is a predisposing factor to Candidiasis and Aspergillosis which is in accordance with earlier findings [26,13]. In this study, out of 9 HIV/mycobacterium spp. co-infected patients, 5 patients were positive of other fungal isolates (table 1). This could mean that HIV/mycobacterium spp. co-infection predisposes individuals to more fungal colonization.

5. CONCLUSION

This study shows that HIV patients in Nnamdi Azikiwe University Teaching Hospital, Nnewi, were often colonized by fungal organisms. These fungal organisms may have contributed to the exacerbated respiratory syndromes in HIV infected patients. The level of CD_4 cell count is also associated with some fungal colonization. High (>400 cells/µl) CD4 counts is suggested to be protective of fungal colonization. More so, antiretroviral therapy has the potency to lower the rate of fungal isolation in HIV patients. Since invasive fungal colonization of the lungs remain important causes of death in immunocompromised patients, early isolation and identification of the colonizing fungi can improve the prognosis of patients.

ETHICAL APPROVAL

All authors hereby declare that the study was examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 declaration of Helsinki.

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COMPETING INTEREST

Authors have declared that no competing interests exist.

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