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Prevalence of Opportunistic Fungal Infections among HIV Infected Patients Attending Antiretroviral Clinic of Federal Teaching Hospital, Abakaliki, Ebonyi State, Nigeria and the Susceptibility of the Fungal Isolates to Selected Conventional Antifungal Drug

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Abstract: Human Immunodeficiency Virus (HIV) does not kill the infected patients directly; instead, it weakens the body's ability to fight disease. The decreased level of immunity in HIV infected patients increases their vulnerability to various opportunistic infections. Opportunistic fungal pathogens have been identified as one of the leading cause of complications and death in HIV/AIDS patients. This research investigated the fungal species associated with the nose and mouth of HIV/AIDS patients in Ebonyi State and also ascertained their susceptibility to three conventional antifungal drugs (itraconazole, nystatin and fluconazole). A total of 120 samples (60 nasal and 60 oral) were collected from 60 HIV positive subjects that enrolled for the study. All cultures were on Sabouraud Dextrose Agar and isolates identified by gram staining, germ tube formation on serum and chlamydospore formation on rice meal Tween 80 agar. CD_4^+ cells count was done by Manual CD_4^+ Cell Counting (Cyto-Spheres). Susceptibility was performed using the agar well diffusion technique. A total of 57 fungal species were isolated (29 from mouth and 28 from nose). *Candida albicans* (42.1%) was the most predominant species isolated, followed by *Aspergillus* species (26.3%), non-albicans *Candida* species (24.6%) and *Cryptococcus neoformans*. (7.0%). Susceptibility test showed that itraconazole had highest efficacy while fluconazole had lowest efficacy. Management of HIV/AIDS patients should involve early laboratory diagnosis of these opportunistic fungal infections and prompt antifungal treatment to reduce the mortality and morbidity associated with the infections thereby enhancing the survival of the HIV infected patients.

Key words: Antifungal Drugs · HIV/AIDS · Opportunistic Infections · Prevalence · Susceptibility Test

INTRODUCTION

HIV/AIDS has been recognized as one of the most devastating infectious diseases to have emerged in recent history [1, 2]. Despite all the efforts to stem this pandemic in most developing countries, its impact is still alarming [3]. Nigeria for instance has second largest HIV epidemics in the world with about 3.6 million people living with the virus in 2016 [4] and approximately 160, 000 people dying of AIDS related illness [5]. Although the HIV is the causative agent of AIDS, it does not kill an individual but its hallmark is the immunosuppression which predisposes to opportunistic infections (OIs) and malignancies [6]. There are global evidences that the overall incidence of

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opportunistic diseases increase with the degree of immunosuppression resulting from HIV disease progression which is signified by the depletion in the CD_4^+ T-cells count [7, 8]. CD_4^+ T-cells count provides a useful prognostic marker in HIV/AIDS and also have critical levels below which certain invasive opportunistic infections starts appearing frequently [9].

Usually, opportunistic infections do not occur in patients with normal CD_4^+ T-cells count of = 1000 cells/µl until the CD_4^+ counts drop to the level = 400-200 cells/µl. Tuberculosis is the most commonly reported opportunistic infection among HIV infected individuals. Nigeria has been ranked among ten countries that together are home to 80% of people living with HIV and tuberculosis co-infection. This tuberculosis epidemic in Nigeria is closely linked with the burden of HIV [10]. Next to tuberculosis is oral candidiasis however, as the CD_4^+ count continues to drop, more endemic and non-endemic opportunistic pathogens invade [8]. Some commonly reported fungal infections in HIV patients are oral candidiasis [11], cryptococcosis, histoplasmosis, aspergillosis etc [9]. Consequently, the complications resulting from the involvement of OIs in HIV/AIDS make the management of HIV/AIDS patients very difficult. Although antiretroviral therapy (ART) is now available for controlling the replication of the virus thereby improving the CD_4^+ counts and reducing the incidence of OIs however, the issues of non-adherence, ART drug resistance and treatment failure hinder the total prevention of OIs among HIV infected patients. Also, in a resource limiting setting like Nigeria, every individual who is eligible to receive ART may not be covered by the government programme and those who are not covered may not afford to buy the drugs on their own. For instance, high rate of HIV associated tuberculosis in Nigeria has been attributed to low antiretroviral treatment coverage of 30% only [12].

Due to the challenges posed by OIs in management of HIV patients, early diagnosis of these infections is vital for better management and preventive measures [9]. Clinical diagnosis of these infections is often presumptive. Therefore laboratory diagnosis is required for conclusive identification and advice for therapy [13]. Although many researchers have highlighted the spectrum of OIs associated with HIV/AIDS [8, 9] however, the opportunistic infection in focus in this study location is tuberculosis with little emphasis on other OIs. Conversely, opportunistic mycoses have been identified as the major player in the morbidity and mortality in HIV/AIDS [14]. Reliable data on the prevalence of opportunistic fungal infections in HIV patients in the study location and the susceptibility profile of the fungal pathogens to commonly used conventional antifungal agents are important for planning and delivery of HIV services such as drug procurement and laboratory services. Therefore, the current study was aimed to investigate the prevalence of opportunistic fungal pathogens among HIV infected patients attending ART clinic of Federal Teaching Hospital, Abakaliki (FETHAI) and evaluation of the susceptibility of the fungal pathogens to some selected conventional antifungal drugs such as fluconazole, itraconazole and nystatin.

MATERIALS AND METHODS

Study Design: The research was undertaken at FETHAI antiretroviral Clinic among HIV seropositive individuals (both in and out patients) attending FETHAI ART clinic. A total of 60 subjects (30 males and 30 females) enrolled for the study and 120 samples (60 oral and 60 nasal) were collected. The samples were analyzed by microscopy and cultural method in the microbiology laboratory of Ebonyi State University, Abakaliki.

Study Population: The study population was all registered HIV sero-positive clients of different educational background and from urban and rural areas attending ART Clinic of FETHAI for medical attention during the study period.

Ethical Approval: Ethical approval was obtained from the research and ethical review committee of FETHAI. The subjects consent was obtained through the clinicians. The subjects were given right to accept or refuse to participate in the study. All the information obtained from the study subjects were kept confidential.

Sample Collection: The oral samples were collected using sterile normal saline to wash the mouth of the subjects into a sterile universal container, while nasal samples were collected with sterile swab sticks. The swab sticks containing the sample were also inserted into a sterile normal saline. Five ml of whole blood were collected from each client into EDTA tubes. The samples were stored in the refrigerator until analysis within 24 hours.

Sample Analysis: All the samples were inoculated on Sabouraud Dextrose Agar (SDA). The oral samples were inoculated by spread plate technique while the nasal samples were inoculated by streaking the swab stick on

the agar plates. The cultures were incubated at room temperature for 48 – 96 h. The morphologically distinct colonies were isolated and repeatedly transferred on SDA plates to obtain pure cultures. The pure isolates were further identified following the method of WHO [9] laboratory guide and mycology - ATLAS.

Identification of Isolates: The pure isolates were identified according to WHO [9] laboratory guide. The *Candida* species were identified by colony appearance on culture, examination of wet mount of the culture, Gram stain, germ tube formation on blood serum and chlamydospore formation on rice meal Tween 80 agar. *Cryptococcus neoformans* was identified by Indian ink stain, urease, nitrate and sugar fermentation tests, while *Aspergillus* species was identified based on the distinct appearance of the colony and microscopic examination (considering the arrangement of the conidia). The identified organisms were kept in SDA slants in bijou bottle and stored in the refrigerator till use.

CD_4^+ Cells Count: The CD_4^+ cells count was done by Manual CD_4^+ Cell Counting (Cyto-Spheres) as described in Iroezindu *et al.* [6]. 100 µl of EDTA blood was placed in a test tube and mixed for 2 min with 10 µl of a monocyte blocking reagent. Thereafter, 10 µl of CD_4 antibody –coated latex spheres were added and the mixture was shaken for 2min. 10 µl of the resultant mixture was transferred to another tube containing 100 µl of lysing reagent. The tube was shaken for 15 s to lyse the red blood cells. Both chambers of a 0.1 mm deep hemocytometer were filled with the mixture and examined under light microscope. Cells bearing three or more latex spheres were counted. The result was multiplied by a factor of 7.3 to account for dilution and also obtain the absolute number of CD_4 T-lymphocyte.

Antifungal Susceptibility Test: The *in-vitro* activity of selected antifungal drugs (nystatin, itraconazole and fluconazole) on the fungal isolates (*Candida albicans*, non-albican *Candida* sp., *Cryptococcus neoformans* and *Aspergillus* sp.) was performed using agar well diffusion technique [15]. The inoculum was prepared using 24 hour culture of *Candida* species and *Cryptococcus neoformans* and 96 hour culture of *Aspergillus* species. The colonies were standardized by suspending it in 0.85% normal saline and comparing the turbidity with 0.5 McFarland standards using spectrophotometer at 530 nm to produce a suspension of $1x10^6$ to $5x10^6$ cells/mL. An aliquot of 0.1 ml of the standardized suspension of the test isolates was inoculated into freshly prepared SDA plates using micropipette and evenly distributed using glass spreader. After the inoculation, 6 mm wells were aseptically made on the agar surface using sterile borer. A stock solution of the antifungal drugs was prepared by tenfold dilution. Micropipette was then used to carefully transfer 0.02 ml of different dilutions of antifungal drugs into the wells. The plates were incubated at room temperature for 24-72 h. The inhibition zone diameters (IZDs) of each of the antifungal agents were measured to the nearest millimeter using meter rule. The minimum inhibitory concentration (MIC) was determined by taking the intercept on concentration axis of the plot of logarithm of concentration against inhibition zone diameter [16, 17].

Statistical Analysis: The fungal carriage in the mouth and nose of HIV/AIDS patients and the number of fungal species in male and female subjects were compared respectively using a t-test analysis. Also, the relationship between the CD_4^+ cells count and the number of fungal species was determined by correlation analysis.

RESULTS

A total of 120 samples (60 oral and 60 nasal) were obtained from 60 HIV sero-positive patients and a total of 57 fungal isolates (28 from nose and 29 mouth) were recovered. *Candida albicans* occurred most predominantly (42.1%). The sex distribution of various isolated fungal species is presented in Fig. 1. The prevalence of different fungal species among various clinical samples is shown in table 1. The commonest species in the oral samples was *Candida albicans* (75%) whereas *Cryptococcus neoformans* occurred most predominantly in nasal sample (75%) followed by *Aspergillus* species (73.3%).

Of all the isolates, *Candida albicans* was more common in female (54.2%) compared to male counterparts (45.8%), but every other isolates studied occurred slightly higher in male subjects however, the differences are not statistically significant ($P>0.05$) (Fig. 2).

The detailed age distribution of various fungal isolates is shown in Table 2. The number of fungal isolates increased with the increase in the age of the patients as observed from the result in the table. Also, the prevalence of HIV infection is higher among the age ranges of 16 - 25 and 26- 35yrs.

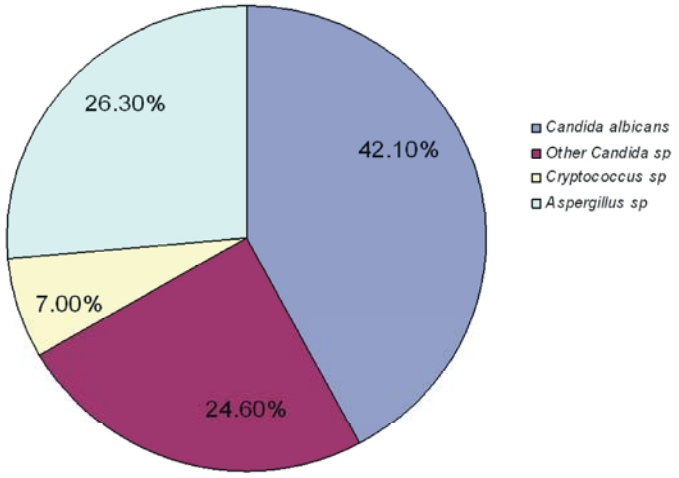

Fig. 1: Distribution of different fungal species isolated from HIV/AIDS patients

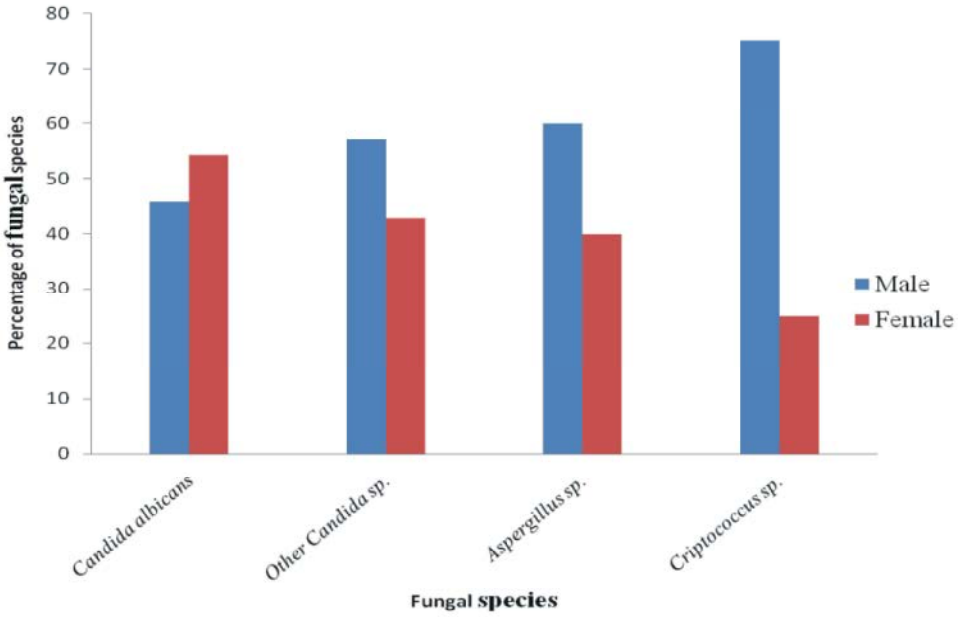

Fig. 2: Sex distribution of fungal species among HIV/AIDS patients

Table 1: Prevalence of different fungal species among various clinical samples

		Distribution in clinical samples (%)	
Number of patients	Fungal species	Oral wash	Nasal swab
60	*Candida albicans*	75	25
	Other *Candida* species	42.9	57.1
	Aspergillus species	26.7	73.3
	Cryptococcus neoformans	25	75

Table 2: Age distribution of fungal species among HIV/AIDS patients

Age range	No of patients	No of fungal species	% of fungal species
6-15	4	3	3.5
16-25	21	26	30.6
26-35	19	28	32.9
36-45	10	19	22.4
46-55	5	9	10.6
Total	85	100	

Table 3: Relationship of CD_4^+ T-cells count with prevalence of fungal species

No of patients	CD_4^+ T cell Range	Mean CD_4^+ T cell	No of fungal sp.	% of fungal sp.
26	80-170	125	35	41.2
22	180-270	225	25	29.4
21	280-370	325	16	18.8
18	480-570	525	9	10.6
Total	85	100		

Table 4: The inhibition zone diameters of the three antifungal drugs on the fungal isolates

Fungal Species	Concentration of antifungal agents (µg/ml) and IZD (mm) Itraconazole							
	10, 000	1, 000	100	10	1	0.1	0.01	0.001
Candida albicans	17	16	14	11	9	6	3	-
Other *Candida* species	21	13	9	8	3	-	-	-
Aspergillus species	23	13	11	7	-	-	-	-
Cryptococcus neoformans	18	16	12	11	8	5	-	-
	Nystatin							
Candida albicans	24	16	13	8	5	2	-	-
Other *Candida* species	24	16	14	6	4	-	-	-
Aspergillus species	19	15	13	-	-	-	-	-
Cryptococcus neoformans	22	16	10	4	-	-	-	-
	Fluconazole							
Candida albicans	43	29	-	-	-	-	-	-
Other *Candida* species	47	25	-	-	-	-	-	-
Aspergillus species	30	7	-	-	-	-	-	-
Cryptococcus neoformans	35	10	2	-	-	-	-	-

Key: IZD = inhibition zone diameter; - = no inhibition

Table 5: The MIC of antifungal drugs on the susceptible fungal isolates

Fungal species	MIC of Antifungal Agents (µg/ml)		
	Itraconazole	Nystatin	Fluconazole
Candida albicans	0.0002	0.005	0.3981
Other *Candida species*	0.0316	0.0501	3.9811
Aspergillus species	0.2512	0.5012	3.1623
Cryptococcus species	0.0010	0.3020	1.0000

Table 3 shows the relationship between CD_4^+ cells count and the number of fungal species. The result showed that the group with lowest CD_4^+ cells count range (80-170cells/µl) had the highest number of species followed by the group with range of 180-270 cells/µl. Correlation analysis showed that the value of CD_4^+ count is directly inversely proportional to the number of fungal species Isolated.

Susceptibility of the Isolates to Conventional Antifungal Agents: The inhibition zone diameter (IZD) of the selected antifungal drugs (itraconazole, nystatin and fluconazole) at different concentrations is shown in Table 4 while the minimum inhibitory concentrations (MIC) of the antifungal drugs are presented in Table 5. It was observed from table 4 that Itraconazole showed a wider range growth inhibition on all the fungal species tested while fluconazole showed a narrow range of growth inhibition. Table 5 showed that itraconazole had lowest minimum inhibitory concentration (MIC) on all the fungal species tested followed by Nystatin, while Fluconazole had the highest MIC. *C. albicans* showed highest sensitivity to all the antifungal agents tested whereas *Aspergillus* species showed highest resistance.

DISCUSSION

Opportunistic infection by some fungal pathogens is among some of the complications commonly encountered in the management of HIV infection. This study highlighted some fungal species particularly yeasts, (*C. albicans,* Other *Candida* species and *Cryptococcus* species*)* and *Aspergillus* species present in the oral and nasal region of HIV sero-positive individuals in the study

location. This is supported by the reports of several studies [13, 18], that have tried to unravel the diversities of fungal opportunistic pathogens associated with HIV/AIDS. The presence of different fungal species colonizing the mouth and nose of these patients is supported by the report of Nnaji *et al.* [19], that diversities of organisms colonize different locations of HIV patient's body as HIV infection progresses to the AIDS status, taking advantage of the suppressed immune system that characterize HIV infection. Also, the observed dominance of *Candida albicans* (42.1%) among the species isolated conforms to the findings of several other researchers [2, 11 & 18]. This might be because *C. albicans* is a member of normal commensal of mouth and nose of even healthy individual, but they can easily take advantage of debilitated immunity to establish infection. This suggests the reason why oropharyngeal candidiasis is common among AIDS patients [2] in that up to 90% of HIV/AIDS patients suffer oropharyngeal candidiasis [20]. The high number of *Aspergillus* species. (26.3%) following *Candida albicans* is in line with the report of Kaur *et al.* [21] which placed *Aspergillus* as the second most commonly recovered fungus in opportunistic mycoses following *Candida albicans* but disagrees with the reports of Jain *et al*. [2] and Parmar *et al*. [22] which placed *Cryptococcus* species second after *Candida albicans* in HIV/AIDS patients. The high number of *Aspergillus* species can as well be due to the endemic nature of the fungus as reported by Hoffman *et al.* [20] that the occurrence of most of these fungal infections is dependent on the endemic nature of the agents.

Cryptococcus neoformans and *Aspergillus* species occurred more predominantly in the nose (75 and 73.3% respectively) than the other fungal species isolated but occurred lower in the mouth (25 and 26.7% respectively). This can be attributed to the fact that nose is their route of entry [11] and they can easily take advantage of the suppressed immune system to establish themselves on the mucous membrane of the nasal cavity, proliferate and add to the number of commensals found in the nose unlike the mouth which is not disposed for inhalation.

The observed increase in the number of fungal species in relation to the increase in the age of the patients is in agreement with the findings of Samie and Mashao [13] where they observed that the prevalence of yeast infections in the respiratory tract was higher among individuals aged above 45 years (77.7%). This could be attributed to the fact that age is a factor in determining the competency of immune system of an individual. This implies that the aged patients are already being challenged by impaired immune system before being infected by HIV which further suppresses their immune system making them more vulnerable to inversion by opportunistic infections. High prevalence of HIV/AIDS found in the age groups of 16 – 25 and 26 – 35 conforms to the report of [19] where they referred this high prevalence in these groups to the fact that they are the most sexually active group and therefore the most vulnerable group to HIV infection. The result is also in consonance with the information in Hoffman, [20], statistical data tables which stated that about 240, 000 adolescents between the ages of 10-19 were living with HIV in 2016 in Nigeria. This number according to the data represents 7% of the total number of persons living with HIV in Nigeria [20].

Several studies have shown that the cardinal feature of HIV infection is the depletion of T-helper lymphocytes (CD_4^+) due to excessive replication of HIV in these cells. It had been reported that the level of CD_4^+ cells counts predicts the likelihood of occurrence and the number of opportunistic pathogens in HIV patients and the chances of having opportunistic infection increases with reduction in the level of CD_4^+ cells count [8]. These reports concurred with the result of this study; the CD_4^+ counts of individuals that enrolled for this study ranged from 80 to 570cells/µl. The result obtained showed number of fungal species isolated to be inversely proportional to the CD_4^+ cells count. The group with lowest CD_4^+ cells count range 80 – 170 had the highest percentage of fungal species (49.1%), followed by the range 180 – 270 (31.6%) and in that decreasing order as the CD_4^+ cells count increases. This agrees with the findings of [23, 24] in which participants with CD4 cell counts of <200 cells/µl were about two times more likely to develop OIs compared to those with CD4 cell counts ≥ 200 cells/µl.

Management of HIV infection involves diagnosis and treatment of complicating factors such as opportunistic infections in addition to the use of anti retroviral therapy. Having observed that fungal infection is one of the complicating factors in HIV/AIDS case, susceptibility test carried out using commonly available antifungal agents showed that Itraconazole had the highest efficacy against the fungal species tested while fluconazole had lowest efficacy. This is in line with the findings of Moges and his colleague in 2016 [26] in which they reported high resistance of yeast species isolated from HIV patients to fluconazole. The result is also in consonance with the report of Samie and Mashao [13] where they observed that resistance to fluconazole and ketoconazole was generally high among all the fungal isolates tested. This shows that itraconazole could be a better option among the conventional antifungal drugs.

CONCLUSIONS

The involvement of opportunistic mycoses in HIV/AIDS complications is an established fact. The result of this study buttressed this fact and also unraveled the presence of different fungal species in the oral and nasal cavities of HIV/AIDS patients. This diversity shows how vulnerable these patients are to the infections resulting from these pathogens. Itraconazole was found to be the best conventional antifungal drug for treating these infections among the three anti-fungal drugs studied. These results suggest the need to include treatment of opportunistic fungal infections in HIV/AIDS management protocol. However, further study is needed to find out the relationship between the occupation and the residential environments of these patients and the diversity of the fungal species to confirm if the occurrence of these species is due to their endemic nature as reported by some researchers. It is also important to know the relationship between CD_4^+ cells count and the susceptibility of isolates to antifungal agents.

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