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ORIGINAL ARTICLE

Oral *Candida* isolates among HIV-infected subjects in Nigeria

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Background: Nigeria is a West African country of more than 150 million persons with the second highest case of HIV/AIDS infected patients in the world. The species spectrum of oral yeast colonization and the susceptibility to a wide range of antifungal agents is poorly understood in Nigeria especially in the south east, south south, and the northern axis. This study evaluates the species spectrum of oral colonization by *Candida* species in HIV-infected patients in Nigeria and the *in vitro* susceptibility pattern of the *Candida* isolates to a broad range of antifungal agents.
Methods: Two hundred oropharyngeal swabs from HIV-infected patients and 100 age-matched healthy controls were screened for yeast isolates using standard procedures and confirmed by the analytical profile index 20C along with other biochemical tests. *In vitro* susceptibility testing of the yeast isolates to antifungals were performed using the broth microdilution method protocol recommended by the Clinical Laboratory Scientific Institute.
Results: Of 200 patients screened, 120 (60%) were colonized by yeasts. *C albicans* was the dominating species in both groups with 54 (45%) isolated from HIV subjects. The non-albicans *Candida* species accounted for 55% with C *tropicalis* 22 (18.3%) showing the highest frequency. We observed that 11.7% of all yeasts isolates were resistant to fluconazole, 8.3% to flucytosine, 7.5% to itraconazole, and 1.7% to voriconazole. All isolates were susceptible to amphotericin B and most of them demonstrated very low voriconazole minimal inhibitory concentrations. Apart from *C albicans*, *C tropicalis* and *C parapsilosis* isolates were also recovered from apparently healthy control subjects.
Conclusion: Although *C albicans* continues to be the dominant *Candida* species in oral *Candida* carriage of HIV-infected patients in Nigeria, the nonalbicans *Candida* species are increasing. Furthermore, the finding of resistant isolates in our study emphasizes the need for antifungal

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susceptibility testing whenever antifungal treatment is desired especially in HIV-infected subjects.

Introduction

Oral candidiasis is caused by *Candida* species colonizing the oral cavity. It is the most common opportunistic fungal infection in individuals infected with HIV and AIDS.[1,2] The risks are considered to be higher in patients with a CD4+ cell count of less than 200 cells/mm^3 and high plasma HIV RNA loads.[3,4] *C albicans* represents the most common causative agent of oral candidiasis; however, other species of *Candida* have begun to emerge.[3] The presence of *Candida* in the oral cavities of HIV/AIDS patients predicts the subsequent development of oral candidiasis. Up till now, no cure exists for HIV/AIDS. It then follows that controlling opportunistic pathogens associated to this pandemic remains one surer way of managing infected individuals who have the scourge. To date, several *Candida* species have been found to be opportunistic especially in HIV/AIDS patients. Among them, *C albicans* remains the most common. However, other species have also been identified in the oropharyngeal cavity of HIV/AIDS patients.[5,6] The incidence of HIV/AIDS has continued to increase globally in recent years especially in developing countries of Africa. For instance, Africa has the world's highest prevalence of HIV infection, with an estimated 24.7 million adults and children older than the age of 15 years living with HIV/AIDS in sub-Saharan Africa alone.[7] Nigeria ranks second after South Africa in the World Health Organization's list of global HIV/AIDS prevalence. In addition, pediatric HIV infection has been increasing in Nigeria over the years, with 74,520 new cases estimated for 2006 alone.[8] With many patients not able to buy antiretroviral drugs or source it from government hospitals free of charge; the death toll has significantly continued to increase. To make matters worse, some nonenlightened individuals who get infected often refuse to admit that they have contracted the virus and rather think that they were given "herbal poisons" by their enemies who live with them in local communities and have continued to distribute this virus among local teenagers. It becomes really difficult to access such patients physically because symptoms, such as loss of weight for both disease situations are similar. Other factors such as illiteracy, lack of good health care facilities, and use of untested herbal medicines have helped to escalate the problem. Although some variety of studies has been carried out on oral candidiasis in Nigeria,[9,10,11] only one of these studies[11] investigated the species spectrum of oral *Candida* colonization in HIV/AIDS patients. However, this study only investigated patients in Lagos, Nigeria and tested only fluconazole resistance of the recovered species. Nigeria is a country of more than 150 million persons with HIV incidence far more in the southeastern axis where our study was undertaken. Our experience with the epidemiology/etiology of other mycotic infections indicates that it varies considerably in different parts of Nigeria apparently because of its socioeconomic and geographical diversity.[12,13] The increasing incidence of HIV/AIDS and the rapid migration of Nigerians to many countries in Asia, Europe, and the United States for business reasons and other purposes requires that the species spectrum of oral *Candida* infections be known so as to provide the necessary and required epidemiological information on the current trend for local and international use by those concerned. Also, the increased incidence of mucosal and probably deep systemic forms of candidiasis has consequently made the use of antifungal agents the best option by clinicians so as to be able to control these pathogens. The widespread use of these antifungals has consequently led to an increase in antifungal resistance. Some authors have further observed a noticeable shift toward nonalbicans species with relative resistance to fluconazole and itraconazole.[14,15] The species spectrum and resistance of *Candida* isolates to currently available antifungal drugs is therefore a highly relevant factor because it causes important implications for morbidity and mortality. Hence, there is a need to carry out *in vitro* susceptibility tests to detect resistant organisms. In the present study, we investigated the asymptomatic oral carriage of *Candida* species in HIV-infected patients in Nigeria and determined the *in vitro* susceptibility pattern of these isolates to amphothericin B, fluconazole, flucytosine, itraconazole, and voriconazole.

Most patients used in the study were sampled at a referral health center, the Ebonyi State University Teaching Hospital, Abakiliki, Nigeria and appropriate permissions were obtained.

Materials and methods

Patients and clinical specimens

A total of 200 oropharyngeal swabs were obtained from HIV-infected patients (108 males and 92 females) in southeast Nigeria. Most of these were from the Ebonyi State University Teaching Hospital Abakiliki, Nigeria. The study lasted for approximately 20 months. Swabs were collected from those aged 19 years and above who were confirmed HIV positive with suspected oropharyngeal lesions/disorders. Swabs were taken from oral lesions when present. The patients were all visiting for the first time. The age- and sex-matched non-HIV subjects were recruited from apparently healthy subjects in the population. Those who had received treatment with antibiotics or antifungal drugs within the last 3 weeks for therapeutic or prophylactic purposes were excluded from this study.

Ninety of these patients were treated with highly active antiretroviral therapy (HAART) that included HIV protease inhibitor (Indinavir) and nucleoside reverse transcriptase inhibitors (Zidovudine, Lamivudine, Nevaripin, or

Stavudine). One hundred (50 for each sex) apparently healthy age-matched subjects were included in the study for comparative purposes. These subjects sampled were not known to have been on any drug in the last 3 weeks before the test. However, some of them admitted to have taken antifungal agents in the past. A structured questionnaire was administered to each patient to obtain relevant data. For the actual collection of specimens, oral swabs were collected with a sterile swab/sterile container respectively and inoculated onto two plates of Sabouraud dextrose agar (SDA) (Difco Laboratories, Detroit, MI, USA) supplemented with chloramphenicol (1 mg/mL) and incubated separately 37°C and 25°C, respectively in ambient air.

Identification of yeast isolates

The identification procedure of yeast isolates was performed as described previously[16] using morphological, microscopic, and biochemical characteristics to confirm cream-colored pasty colonies exhibiting characteristic yeast smell with large gram-positive cocci, which initially grew on SDA. Any multiple colonies were separately identified. The identified isolates were confirmed by the analytical profile index 20C (BioMerieux, Marcy L' Etoile, France). Chromagar (Chromagar, Paris, France) was used for all isolates and discrimination tests between *C albicans*, *C dubliniensis*, and other species were investigated by analysis of germ tube formation in calf serum at 37°C for 3 hours, degree of chlamydospore production on cornmeal agar supplemented with 1% Tween-80, colony morphology was on Staib agar, growth at 45°C on SDA, and xylose assimilation test was carried out as previously described by other authors.[16,17] Two typed isolates *C albicans* American Type Culture Collection (ATCC) 10231 and *C dubliniensis* ATCC 777 were included as control.

Antifungal susceptibility testing

In vitro susceptibility testing of the yeast isolates were performed using the broth microdilution method protocol recommended by the Clinical Laboratory Scientific Institute (formerly NCCLS).[18] Amphotericin B (Bristol-Myers Squibb, Princeton, NJ, USA), itraconazole (Jansen Pharmaceuticals, Beerse, Belgium), voriconazole, flucytosine (Sigma, St Louis, MO, USA), and fluconazole (Pfizer International, New York, NY, USA) were tested against all the yeast isolates recovered from the study. The final concentrations of drugs ranged from 0.06 μg/mL to 64 μg/mL for fluconazole and flucytosine; from 0.015 μg/mL to 16 μg/mL for amphotericin B, itraconazole, and voriconazole. The medium used was Roswell Park Memorial Institute 1640 broth with L-glutamine without bicarbonate (GIBCO BRL, Life Technologies, California, USA) buffered to pH 7.0 with 0.165 M 3-*N* morpholinepropanesulfonic acid (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and sterilized by filtration. The suspension of yeast cultures after 48 hours of incubation was prepared in sterile normal saline (0.85%) adjusted using spectrophotometer at 530 nm to match the turbidity of a 0.5 McFarland standard. This was then diluted in Roswell Park Memorial Institute 1640 media to obtain a final concentration of 1×10^3—5×10^3 colony forming units/mL. The microplates were incubated at 35°C for 48 hours. Minimal inhibitory concentrations (MICs) were defined for amphotericin B as the lowest concentration of drug, which resulted in a complete inhibition of visible growth, whereas for the three azoles and flucytosine, it was defined as the lowest concentration of drug that produced a 50% reduction in fungal growth compared with that one of drug-free growth control as in Clinical and Laboratory Standards Institute (CLSI) guidelines.[18] Two quality control isolates, *C albicans* ATCC 90028 and *C parapsilosis* ATCC 22019, were included on each day of testing to check the accuracy of drug dilutions and the reproducibility of the results. The purity and viability of all tested organisms were checked by subculturing the inoculum suspension on SDA. For determination of resistance, interpretive susceptibility criteria for fluconazole and 5-flucytosine were those recommended by the CLSI. For fluconazole, isolates showing MICs $\geq$64 μg/mL were regarded as resistant, whereas for 5-flucytosine, isolates showing MICs of $\geq$32 μg/mL were regarded as resistant. For voriconazole, $\geq$4 μg/mL was recently approved as the CLSI breakpoint for resistance.[19,20] Because of the lack of defined breakpoints for amphotericin B, isolates showing MICs of $>$1 μg/mL were considered as resistant. The Chi-squared test was applied to analyze correlations of *Candida* species with antiretroviral therapy and between infected and noninfected subjects. A p value less than 0.05 was considered statistically significant.

Results

Of 200 patients investigated, 120 of them (90 treated with HAART) were colonized with different kinds of yeast. Detectable growths were produced by all isolates after incubation for 24—48 hours. *C albicans* had the highest frequency among all the species with a total number of 54 (45%). The nonalbicans species and their frequencies included *C tropicalis* 22 (18.3%), *C parapsilosis* 18 (15%), *C guilliermondii* 11 (9.2%), *C dubliniensis* 9 (7.5%), *C lusitaniae* 2 (1.7%), *C krusei* 2 (1.7%), and *C kefyr* 2 (1.7%). *C albicans* was isolated in 50 (55.6%) patients treated with HAART and in 23 (76.7%) patients who were not on HAART. In the age-matched control subjects, *C albicans* was the major species recovered and comprised 13 and 12 isolates from male and female subjects, respectively (Table 1). They were all sensitive to all the five anifungals tested against them (data not shown). *C tropicalis* and *C parapsilosis* were also recovered from the controls subjects. Similarly, for the nonalbicans *Candida*, 40 (44.4%) were isolated from patients treated with HAART, whereas 7 (23.3%) isolates were from patients who were not on HAART. No significant statistical association was found between antiretroviral therapy and species of *Candida* isolated in the study ($p = 0.110$). There was no significant difference between the occurrence of the species and sex of both infected and noninfected subjects ($p > 0.05$). For the 120 yeast strains, the MIC ranges were 0.015—16 μg/mL for itraconazole, 0.125—64 μg/mL for fluconazole, 0.002—$\geq$32 μg/mL for flucytosine, 0.015—8.0 μg/mL for voriconazole, and 0.015—1.0 μg/mL for amphotericin B.

Table 1 Age and gender distribution of HIV and non-HIV infected subjects screened in the study

Age (yr)	Male		Female		Total	
	HIV infected, *n* (%)	Noninfected, *n* (%)	HIV infected, *n* (%)	Noninfected, *n* (%)	HIV infected, *n* (%)	Noninfected, *n* (%)
19–29	27 (25)	13 (26)	14 (15.2)	8 (16)	41 (20.5)	21 (21)
30–39	25 (23)	12 (24)	29 (31.5)	14 (28)	54 (27)	26 (26)
40–49	23 (21.3)	10 (20)	25 (27.2)	16 (32)	48 (24)	26 (26)
50–59	21 (19.4)	10 (20)	15 (16.3)	9 (18)	36 (18)	19 (19)
>60	12 (11.1)	5 (10)	9 (9.8)	3 (6)	21 (10.5)	8 (8)
Total	108 (100)	50 (100)	92 (100)	50 (100)	200 (100)	100 (100)

The isolates demonstrated very low voriconazole MICs, in which 80% presented values of 0.015 μg/mL. For using the CLSI suggested MIC interpretive criteria, we found that 14 (11.7%) of all yeasts isolates were resistant to fluconazole, 9 (7.5%) to itraconazole, 10 (8.3%) to flucytosine, and 2 (1.7%) to voriconazole. All *Candida* isolates were susceptible to amphotericin B. *C albicans* was the most resistant species (17.5%) followed by *C parapsilosis* (7.5%) and *C tropicalis* (4.2%). On the other hand, *C kefyr* and *C lusitaniae* isolates showed low MICs to azoles and to amphotericin B. However, *C krusei* particularly showed high MICs to flucytosine and fluconazole. The MIC ranges, MICs at which 50% (MIC_{50}) and 90% (MIC_{90}) of the strains were inhibited and resistance to itraconazole, fluconazole, flucytosine, amphotericin B, and voriconazole for all *Candida* isolates tested are shown in Table 2. The MIC ranges for the reference strain *C albicans* ATCC 90028 and *C parapsilosis* ATCC 22019 were within the reference range stipulated in the CLSI guideline (data not shown).

Discussion

Little is known about the etiological importance and the species spectrum of different yeast species in the oral colonization and infection among Nigerian HIV/AIDS patients especially in the southeastern axis where the population is high with higher cases of HIV/AIDS. This study was aimed at finding out the species spectrum of *Candida* species colonizing the oral cavity in HIV/AIDS patients and

Table 2 Antifungal susceptibility profile of 120 oral Candida isolates from HIV patients

Species	Antifungal agents	MIC range	MIC_{50}	MIC_{90}	Resistant number (%)
C albicans ($n = 54$)	Amphotericin B	0.015–0.5	0.125	0.5	—
	Itraconazole	0.015–16	0.03	0.5	6 (11.1)
	Voriconazole	0.015–8.0	0.015	0.03	1 (1.9)
	Fluconazole	0.125–64	0.5	64	9 (16.7)
	Flucytosine	0.12–≥32	0.12	1.0	5 (9.3)
C tropicalis ($n = 22$)	Amphotericin B	0.015–0.25	0.03	0.0125	—
	Itraconazole	0.015–0.25	0.015	0.125	—
	Voriconazole	0.015–1.0	0.015	0.015	1 (7.7)
	Fluconazole	0.25–64	4.0	4	—
	Flucytosine	0.12–≥32	0.5	1.0	2 (9.1)
C parapsilosis ($n = 18$)	Amphotericin B	0.015–0.5	0.125	0.5	—
	Itraconazole	0.015–2.0	0.03	0.5	1 (8.3)
	Voriconazole	0.015–0.5	0.015	0.0125	—
	Fluconazole	0.125–64	1.0	4.0	2 (11.1)
	Flucytosine	0.25–≥32	1.0	2.0	2 (11.1)
C guilliermondii ($n = 11$)	Amphotericin B	0.06–1.0	0.25	1.0	—
	Itraconazole	0.5–16	0.5	16	1 (33.3)
	Voriconazole	0.015–1.0	0.015	0.5	—
	Fluconazole	0.5–8	1.0	8.0	—
	Flucytosine	0.12–≥32	0.12	8.0	—
C dubliniensis ($n = 9$)	Amphotericin B	0.015–0.5	0.125	2.0	—
	Itraconazole	0.015–16	0.015	1.0	1 (11.1)
	Voriconazole	0.015–8.0	0.015	1.0	—
	Fluconazole	0.125–64	2.0	4.0	3 (33.3)
	Flucytosine	0.12–16	1.0	8	1 (11.1)

MIC defined as the lowest concentration, which resulted in no growth for amphotericn B and 50% reduction in turbidity for flucytosine, itraconazole, fluconazole, and voriconazole; MIC_{50} and MIC_{90}: MIC value was able to inhibit 50% and 90% of the isolates tested, respectively.
MIC = minimal inhibitory concentration.

the antifungal susceptibility profile of the isolated species to a wider spectrum of antifungal agents. A recent study in Lagos, Nigeria investigated oral yeast colonization but tested the recovered isolates for only fluconazole resistance.[11] Our finding of *C albicans* as the predominant species is consistent with the above report but there were some striking differences in the species spectrum and percentage of recovered isolates. For instance, we recovered a total of 54 (45%) for *C albicans*, 22(18.3%) of *C tropicalis*, 18 (15%) of *C parapsilosis*, and 11 (9.2%) of *C guilliermondii*. Theirs were respectively lower: 30(40.5%) for *C albicans*, 13(17.6%) for *C tropicalis*, 3(4%) for *C parapsilosis*, and 1(1.4%) for *C guillermondii*. Similarly, their study apparently could not isolate *C dubliensis* but this species makes up about 7.5% of the isolates we recovered. Although we have no immediate reasons for this observation, the level of diversity and other differences among the various population of more than 150 million in different regions of Nigerian could be responsible. We also observed a similar trend (different percentage distribution and species spectrum) in the etiology and prevalence of dermatological infections among different geographical regions of Nigeria.[12,13] Another difference is our finding of 11.7% of *Candida* species resistant to fluconazole compared with 9.5% in Lagos. Unfortunately, we are not able to compare the resistance patterns to the rest of the other drugs because their study tested only fluconazole susceptibility. However, our findings on *C albicans* as the most prevalent species agrees with other published reports from different parts of the world.[5,21,22] Although this contrasts with the observation in some countries in the Gulf region,[23] it is similar to the findings reported from Brazilian HIV/AIDS patients.[5] In Europe, North America and Australia, *C albicans* usually accounts for 60–80% of yeasts isolated from the mouth of healthy persons. However, oral mycobiota varies from country to country and *C albicans* is not always the predominant species.[15] Studies of epidemiologic surveillance have showed that the proportion of non-albicans species is increasing among the HIV-infected patients.[5] In our study, although *C albicans* was the predominant species, the nonalbicans species represented 55% of all the isolates recovered in the study. This is much higher than 42.1% reportedly isolated from the same category of patients in Brazil.[5] Nonalbicans species play an important role as causative agents in oropharyngeal candidiasis and have been associated with severe symptoms.[5] Medicines, such as antibacterial antibiotics, antiretroviral, and antifungal agents can interfere in changes in the species distribution.[18] In patients who have not taken protease inhibitors, *C albicans* has been most common than nonalbicans.[5] This is consistent with our finding of *C albicans* as the major species recovered from the age-matched control normal subjects. We have noted that although the percentage of *C albicans* isolates has been higher in non-treated patients with HAART than in the treated ones, there were no significant association between antiretroviral therapy and *Candida* species ($p = 0.110$). Although the frequency is different, the isolation of *C dubliniensis* in our study is similar to the published observations in the United States and Brazil[5,22,24] but in contrast to another Brazilian study.[5] We were unable to isolate *C glabrata* that has been considered an emerging pathogen. Although it was recovered in the Lagos study, it constituted only a small fraction (5.4%) of the total isolates recovered in that study, suggesting that it may not be very common in the oral flora of HIV-infected subjects in Nigeria. It may be difficult to explain the reason for the variability in our frequency of the *Candida* species with those of other authors.

Our result on *in vitro* susceptibility of *Candida* species to amphotericin B is consistent with those reported previously[5,25] and shows that *Candida* isolates recovered in the study were highly sensitive to polyene antifungal, amphotericin B. The MIC distribution was concentrated in a very narrow range. Although it has been suggested that *C lusitaniae* isolates may present innate resistance to amphotericin B,[5] the two recovered *C lusitaniae* isolates showed MIC of 0.25 μg/mL and was categorized as susceptible to amphotericin B according to the interpretative breakpoints adopted in our study. These results confirm those of Pfaller et al.[19] who noted that primary resistance to amphotericin B was not very common among *C lusitaniae* isolates. The introduction of effective antiretroviral therapy has no doubt made a tremendous impact on the natural history of HIV infections and its management, especially reducing the incidence of oropharyngeal candidiasis and a trend toward less-frequent *in vitro* resistance to fluconazole.[5] Unfortunately, our study showed that fluconazole resistance was relatively high in the HIV-infected group. We found that 12.31% of *Candida* isolates were resistant *in vitro* to fluconazole (MIC $\geq$ 64 μg/mL). Conversely, all the species recovered from the age-matched control groups were susceptible to all the antifungals tested. Factors, such as the degree of immunosuppression of the patients, the chemotherapeutic drugs use, and the intrinsic resistance of *Candida* species[5] may contribute to fluconazole resistance. In our study, 15 (16.7%) of the isolates obtained from 90 patients who used HAART were resistant to fluconazole and 4 (13.3%) of the isolates identified from 30 patients who did not receive HAART were resistant to this drug. *Candida* isolates were generally more susceptible to voriconazole than to itraconazole. Also, 12.5% of our isolates were resistant to flucytosine. Both observations agree with those ones verified by other researchers.[5,15,22]

Despite the fact that *C albicans* continues to be present in most yeast carriage from oral mucosa of HIV-infected patients in Nigeria, its frequency may vary, with a recent trend to nonalbicans species. Our study shows that the species of nonalbicans were isolated in 55% of the patients. All isolates were susceptible to amphotericin B, which has been the first choice of treatment for severe fungal infections for more than several decades. However, its use has been limited by a number of serious adverse effects. Voriconazole presented potent activity against *Candida* sp, including those that were resistant to fluconazole and itraconazole isolates. Because voriconazole is well tolerated, it could be used against *Candida* infections caused by strains resistant to fluconazole and itraconazole. Unfortunately, this drug is not very commonly available in Nigeria and accessibility may be poor. We agree that surveillance programs are needed to identify possible changes in the species distribution and antifungal susceptibility patterns of yeasts peculiar to each region or country as a way of monitoring the trend and ensuring better management of HIV/AIDS patients.

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