Fluconazole resistant opportunistic oro-pharyngeal candida and non-candida yeast-like isolates from HIV infected patients attending ARV clinics in Lagos, Nigeria.

C.A. Enwuru[1]; A. Ogunledun[2]; N. Idika[1]; N.V. Enwuru[3]; F. Ogbonna[1], M. Aniedobe[1], A. Adeiga[1]

1. Nigerian Institute of Medical Research, Yaba, Lagos, 2. Department of Microbiology and Parasitology, School of Post Graduate Studies Olabisi Onabanjo University, Ogun State, Nigeria, 3. Faculty of Pharmacy, Lagos University Teaching Hospital (LUTH), Idiaraba Lagos, Nigeria.

Abstract

Background: Oro-Pharyngeal Candidiasis (OPC) continues to be considered the most common opportunistic fungal disease in HIV/AIDS patients globally. Azole antifungal agent has become important in the treatment of mucosal candidiasis in HIV patients. Presently, antifungal drug resistance is fast becoming a major problem particularly with the immune depleted population.

Objectives: This study was designed to investigate the: existence of OPC, species distribution fluconazole susceptibility profile of yeast cells isolated from oral specimens of HIV/AIDS patients from Lagos Nigeria, between Oct. 2004 and June, 2005.

Methodology: The venous blood samples were screened for HIV antibodies using the Cappillus HIV I and II test kit (Trinity Biotech Plc UK), and Genie II HIV I and II EIA kit (Bio-Rad France). The positive results were subsequently confirmed at the laboratory attached to each of the clinics, using the Nigerian Federal Ministry of Health approved algorithm. The samples from 213 (108 females and 105 males) HIV positive patients were plated onto SD agar. The isolates were identified by morphotyping, microscopy and speciated using germ tube test and battery of biochemical sugar fermentation and assimilation tests. Fluconazole agar diffusion susceptibility testing was carried out on each isolates.

Results: Seventy-four (34.7%) isolates were recovered including one person with double isolates. Only 70(94.6%) of the isolates could be adequately speciated. *Candida albicans* 30 (40.5%) was the most frequently isolated species, the rest were non-albicans species, with the frequency of *C. tropicalis* › *C. Krusei* › *C. glabrata* and *C. neoformans* for species for species having up to 4 isolates. Four (30.8%) out of 13 isolates of *C. tropicalis* showed germ tube formation. While one *C. albicans* was germ-tube negative. Out of the 74 isolates tested for fluconazole sensitivity, 58(78.4%) were sensitive, MIC d" 8µg/ml, 9(12.1%) were susceptible Dose Dependant (S-DD), MIC 16-32 µg/ml and 7(9.5%) were resistant, MICs e" 64µg/ml. Among the *C. albicans* isolates, 26(86.7%) were sensitive to fluconazole. The rank of susceptibility was *C. albicans* > *C. tropicalis* > *C. Krusei* for the most prevalent species.

Conclusion: We conclude that fluconazole resistant strains of oro-pharyngeal yeast-like cells exist in about 9.5% of HIV/AIDS patients with the above stated species distribution. We therefore, highlight the need for routine antifungal susceptibility testing on HIV patients with cases of initial or repeat episodes of OPC.

Key words: Oropharyngeal Candida (yeast-like cells), HIV/AIDS and Fluconazole Resistance.

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Introduction

Oro-Pharyngeal Candidiasis (OPC) is the most commonly reported opportunistic infection observed in HIV/AIDS patients, occurring in an estimated 80-95% of those with HIV disease[1, 2]. Human Immunodeficiency Virus (HIV) works by weakening the body's immune system[3]. Increase in retroviral replication and an associated decline in immune defenses render these 'at risk' patients particularly susceptible to OPC[4]; to the extent that OPC (oral thrush) is considered a strong indication of HIV associated immunodeficiency. The prolonged nature of AIDS predisposes these subjects to repeat episodes of OPC, which can increase in frequency and severity with progressive immune depletion as the disease progresses[3].

Candida albicans is the most implicated, Ehrahim[5] and Dunic[6] reported prevalence levels of 52.4% and 77.7% respectively from HIV/AIDS patients.

Fluconazole, is considered the drug of choice for the treatment of the most common HIV associated opportunistic yeast infections[7]

Antifungal drug resistance is fast becoming a major problem; particularly with the immuno-compromised population[8]. The increased reports of antifungal resistance and expanding drug therapy options prompted the need for clinically relevant antifungal

Correspondence:
C.A. Enwuru.
Microbiology Division,
Nigerian Institute of Medical Research,
P.M.B. 2013, Yaba, Lagos -Nigeria.
E-mail enwuru100@yahoo.ca

susceptibility testing[9]. Fluconazole resistance is associated with prolonged exposure to azoles[9]. Many studies have estimated the incidence of clinical fluconazole resistance to be from: 6 - 36% [11], 15%[12] and 5-10%[13]. Espinel-Ingroff[14] reported an association between in vitro resistance and clinical failure. The aims of this study were to determine the prevalence of OPC, species distribution, and to analyze the in vitro analyze the invitro susceptibility profile of the isolates against fluconazole – the most frequently used anticandidal agent against OPC in the three ARV clinics in Lagos Nigeria.

Materials and methods

The study was conducted between Oct. 2004 through Dec., 2005 at the Nigerian Institute of Medical Research (NIMR), microbiology laboratory. Samples were collected from 3 Anti Retro Viral (ARV) Clinics (NIMR, Lagos University teaching Hospital (LUTH) and the Infectious Disease Hospital (IDH), Lagos. Lagos is a metropolitan heterogeneous Nigerian society; about 15 million people live in it, with various tendencies to the high-risk behaviours associated with HIV/AIDs pandemic.

Selection criteria of the patients: The patients selected for the study were visiting the clinic for the first time and confirmed positive of HIV, presented with oro-pharyngeal disorders symptoms, were 18 years and above, male or female, were not on any antifungal therapy at least 3 weeks before recruitment. All patients that met the inclusion criteria were the only ones documented and assigned serial referral numbers.

Questionnaire: For each registered patient a structured questionnaire was administered in a private room after obtaining his or her informed consent. The questionnaire was designed to obtain information on demographics (bio data), date of first diagnosis, clinical and laboratory history of oro-pharyngeal candidiasis, number of repeat episodes (An episode was defined as a case of history of noticeable oral thrush which was resolved after antifungal chemotherapeutic intervention), date of commencement of present episode and any previous suspected case of fluconazole resistance via the referral note.

Sample collection and processing

Venous blood samples were screened for HIV I and II antibodies using the Cappillus Test Kit (Trinity Biotech Plc, UK). The positive results were further screened using Genie II kit (Bio-Rod, France), further confirmatory tests were carried out at the various clinics' laboratories, using approved standards. All the patients who consented to the study were all positive for HIV when the screening results were matched with the confirmatory results.

Sputum or oral swabs/ scrapings were collected from each patient in a wide mouthed screw capped, leak-proof container (sputum), the oral swabs with 'Evepon' sterile swab stick (Evepon Industries Ltd. registered as NAFDAC NO 03-0482 Nigeria) or the scrapings with sterile spatula in a sterile universal container. Samples were inoculated onto two plates of sabouraud Dextrose (Difco Laboratories, Detroit) 2% glucose agar supplemented with chloroamphenicol (50mg/l)[15,16] ,and incubated one at room temperature (25-28°C) and the other at 37°C, first reading was done after 24 hours and subsequently for a period not exceeding five days.

Identification: Growth on SDA was identified by cultural characteristics, morphotyping, microscopy and battery of biochemical tests.

On a sabouraud agar, cream-coloured pasty colonies with characteristic yeast smell whose Grams reaction appears large (3-6m by 6-10m) positive cocci were subsequently confirmed to be yeast cells[17]. Any multiple colonies were separately identified.

Speciation: Germ tube procedure[18] was conducted on each isolate. Sugar (glucose, galatose, arabinose, maltose, lactose and sucrose) fermentation was described as the production of gas or bobbles and possibly a colour change[17, 19]; concurrently, Sugar assimilation procedure were conducted; using minimal sugar-free nitrogen containing basic agar, inoculated with highly turbid suspension of overnight subculture of each yeast-like cells, the preparation was allowed to set, dried and previously sugar-impregnated-discs were placed on the plates, incubated overnight. A milky halo formation around each disc corresponds to diffusion zone of that particular sugar and hence its assimilation.

Where necessary, organisms were also checked for urease production, nitrate assimilation, and ascospore formation[20].

Antifungal susceptibility profile

Susceptibility to fluconazole was tested using NCCLS M27-A recommended macro dilution method[21.] Reference grade powder marked 'physicians' sample' of fluconazole was obtained from Pfizer pharmaceuticals Anglophone West Africa Ltd, Ikeja Nigeria. The final drug concentrations obtained were between 0.625 – 64 mg/ml.

The inoculum suspension was adjusted by the spectrophotometer to that produced by a 0.5 Mc Farland Standard at 530nm wavelength to produce 1 x 10^6 to 5 x 10^6 cells per ml. A working suspension was made by a 1: 100 dilutions followed by a 1:20 dilution of the stock suspension resulting in 5.0 x 10^2 to 2.5 x 10^3 cells per ml[21].

In place of ATCC 22019 *C. Parapsilosis* quality control strain recommended for quality control by NCCLS *C. Parapsilosis* previously isolated from one AIDS patient that responded to a single dose fluconazole treatment from the ARV clinic without a repeat episode in 6 months period and which was completely inhibited by NCCLS MIC dilutions was used as control and was included on each day of the assay to check the accuracy of the drug dilutions and the reproducibility of the result.

Within 15 minutes of inoculum standardization, 0.9ml of the adjusted inoculum was added to each tube in the dilution series and mixed with 0.1ml of various antifungal concentrations (i.e. 1:10 dilution).

Control: The growth control received 0.1ml of drug diluent (sterile distilled water) without the antifungal agent.

Guide to turbidity reading

The amount of allowable turbidity was estimated by diluting 0.2ml of drug-free control growth with 0.8ml of broth medium producing 80% inhibition standard.

The Minimal Inhibitory Concentration (MIC) of the fluconazole is defined as the lowest drug concentration, which resulted in 80% decrease in turbidity as compared with that in the growth control (drug-free) and the allowable turbidity standard[21].

Breakpoint definitions for fluconazole were those published by the NCCLS M27 A[21], and were as follows: susceptible MIC d" 8µg/ml, Susceptible – Dose Dependent (S-DD) MIC = 16 - 32µg/ml and Resistance ® MIC e" 64 µg/ml.

Ethical consideration

This study was approved by the Nigerian Institute of Medical Research (NIMR) Institutional Review Board (IRB). The approval was an adequate pass to other settings where the study took place.

Informed consent was obtained from each subject in an enclosure, after the goals and objectives of the study were explained to them. Absolute confidentiality was maintained and the risk/benefit analysis of the study was adequately highlighted in the language each participant understood. The results were released to them for their clinical care at no cost.

Result

A total of 73(34.3%) out of the 213 (108 female and 105 males) patients with OPC enrolled in the present study had yeast like cells recovered from their samples. With only one person harbouring more than one species bringing the total number of candidal isolates to 74. The age and gender distributions of the study population were recorded in table 1.

Table 1: age and gender distribution of 213 HIV/AIDS patients presented with broncho-oropharyngeal symptoms attending the ARV clinics in Lagos, Nigeria.

Age (years)	Male N (%)	Female N (%)	Total N (%)
18 – 28	03(2.90)	39(36.10)	42(19.70)
29 – 39	39(37.10)	51(47.20)	90(42.20)
40 –50	42(40.0)	15(13.90)	57(26.80)
51 – 61	18(17.10)	03(2.80)	21(9.90)
>62	03(2.90)	00(00)	03(1.40)
Total:	105(100%)	108(100%)	213(100%)

The incidence of different species amongst the 74 isolates is show in table 2. Only 70(94.6%) of the yeast isolates could be speciated using the battery of biochemical tests stated earlier.

Thirty (40.5%) of the isolates were *Candida albicans*, the rest were Non Candida Albicans Candida (NCAC) species, with the frequency of *C. tropicalis* 13(17.6%)> *C. krusei* 5(6.8%) > *C glabrata* 4(5.4%); others are as shown in table 2. Only 3(4%) showed indeterminate variation from the classical reaction and therefore, could not be completely speciated. Four strains of *C. tropicalis* and the suspected *C. dubliniensis* showed germ tube formation after incubating with human plasma. One strain of *C. albicans* did not show germ tube formation even when repeated.

Table 2: descending number of various yeast species isolated from HIV/AIDs broncho-oropharyngeal samples.

Various species	Number isolated (%)
Candida albicans	30(40.50)
Candida tropicalis	13 (17.60)
Candida krusei	05(06.80)
Candida glabrata	04(05.40)
Cryptococcus neoformans	04(05.40)
Candida pseudotropicalis	03(04.00)
Candida parasilosis	03(04.00)
Candida famata	03(04.00)
Candida kefyre	02(02.70)
Candida gulliermondii	01(01.40)
Rhodotorula rubbra	01(01.40)
Trichosporon cutaneum	01(01.40)
Candida dubliniensis?	01(01.40)
Indeterminate	03(04.00)
Total:	74(100%)

The fluconazole sensitivity profile conducted had 58(78.4%) of the isolates sensitive MICs d" 8µg/ml, 9(12.1%) were susceptible Dose Dependant (S-DD), MICs 16-32 µg/ml, and only 7(9.5%) were resistant MICs e" 64 µg/ml, table 3.

Table 4 shows various candida species studied and their susceptibility profile to fluconazole. The rank of susceptibility was *C. albicans* > *C. tropicalis* > *C. krusei* for those having more than 5 isolates.

Out of the 30 *Candida albicans* isolated, 26(86.7%) were sensitive; one (3.3%) was S-DD. while 11(84.6%) out of the 13 *Candida tropicalis* were susceptible. All the indeterminate species (n=3) were sensitive. Among the 7 resistant isolates, 3 were *C. albicans*, 2 *C. Krusei,* 1 *C. tropicalis* while the supposed *C. dubliniensis?* Was also, non-sensitive.

Discussion

This result has shown that broncho-oropharyngeal candida and non-candida yeast infection is presently an important opportunistic disease amongst HIV/AIDS patients. The present study revealed 34.2% prevalence of oral yeast infections within the population studied(those co-infected with or solely with opportunistic organisms other than yeast-like cells were recorded in a different study). This was lower than the 84% reported byNeil[22]. Dunic[6] reported 77.7% from Serbia; while Reichart[23] reported 48% in Thailand and Cambodia, all from adult population. The higher prevalence reported by these previous workers could be ascribed to the general report of substantial rise in oral candidiasis between 1990 to 1999 from England and Wales[24].

In the present study, 94.6% of the yeast-like cells isolated could be identified and speciated-using battery of biochemical tests cited. The result obtained is comparable to the previous reports of 98.8% by Dolan[25] and 96.0% by Huppert[26], but higher than 76.0% reported by Taschjian[17].

Candida albicans (40.5%) was the most frequently isolated species; this agrees with the findings of Ehrahim[5] and Rejane[16,] who reported 52.4% and 57.4% respectively. Jabra-Rizk[8] reported that all species of Candida isolated from the oral thrush of people living with HIV or having AIDS are potentially pathogenic. However, the knowledge of the species isolated is imperative since some species of yeast cells are known to be intrinsically resistant to some antifungal drugs, e.g. *Candida krusei* to fluconazole[16].

In this study of HIV/AIDS patients with OPC, *C. albicans* is followed in frequency by *C. tropicalis*, *C. krusei*, *C. glabrata*, *C. pseudotropicalis*, *C. parapsilosis* and other non albicans spp.; Compare with the frequency reviewed: *C. albicans* was frequently followed by *C. glabrata, C. tropicalis*, *C. krusei*, *C. parapsilosis*[1, 28]. From all the above stated reports, one can deduce that the prevalence of oral candidiasis in HIV/AIDS patients varies with the environment, although *C. albicans* remains generally the most implicated.

From the germ tube rapid test, the present study reports four strains of *Candida tropicalis* that had germ tube formation, and one strain suspected to be *Candida dubliniensis*? Again one strain of *C. albicans* did not show any germ tube formation. Tierno and Milstoc[29] reported 26 strains of germ tube positive *C. tropicalis*. Again Calderone[20] wrote that formation of germ tubes in serum or similar media is characteristic of 95 to 97% of clinical isolates of *C. albicans* and *C. dublinensis*. From the present findings, the reliability of germ tube production as a confirmatory test for *Candida albicans* in HIV infection was as high as 96.7% and is therefore recommended for continued use.

Azoles (fluconazole in particular) are considered the drug of choice for treating oral candidiasis associated with HIV/AIDS patients[7] Secondary and prolonged exposure was highlighted as the main cause of emergence of resistance to azoles seen over the few years[8]. Sangeorzan[30], reported that MIC of fluconazole leads to both clinical treatment failure and antifungal resistance. Gabriel[31], Maenza[10] and Andrew[3] reported that apart from prolonged exposure, advanced immuno suppression is a major risk factor for fluconazole

Table 3:The MICS of macrodilution procedure conducted

Sensitive MIC d" 8µg/ml				S-DDMIC › 8 - 32µg/ml		ResistantMIC ≥ 64µg/ml	
P-Code	MIC	P-Code	MIC	P-Code	MIC	P-ode	MIC
MR/10/01	0.625	MR/1/43	8.0	MR/10/02	32	LU/10/08	>64
MR/10/03	0.1	ID/1/45	0.25	MR/10/06	>8	LU/11/17	-
MR/10/04	0.25	ID/1/46	1.0	MR/12/27	>8	LU/11/20	>64
MR/10/05	0.25	ID/1/47	1.0	MR/11/33	32	MR/12/23	-
MR/10/07	1.0	ID/1/48	0.25	LU/12/36	32	MR/12/26	-
LU/10/09	4.0	ID/1/49	1.0	MR/11/44	32	MR/2/58	-
LU/10/10	1.0	ID/1/50	8.0	ID/1/51	>8	LU/12/66	-
MR/11/11	0.25	ID/1/52	0.25	MR/2/59	32		
MR/11/12	0.625	ID/1/53	1.0	MR/2/60	32		
MR/11/13	4.0	LU/1/54	8.0				
MR/11/14	1.0	LU/1/55	8.0				
MR/11/15	4.0	LU/1/56	0.25				
MR/11/16	1.0	LU/1/57	0.625				
LU/11/18	1.0	MR/2/61	4.0				
LU/11/19	0.25	MR/2/62	1.0				
LU/11/21	4.0	LU/2/63	1.0				
LU/11/22	0.25	LU/2/64	4.0				
MR/12/24	1.0	LU/2/65	1.0				
MR/12/25	0.25	ID/2/67	0.25				
MR/12/28	4.0	ID/2/68	1.0				
MR/12/29	1.0	ID/2/69	4.0				
MR/12/30	4.0	ID/2/70a	0.25				
MR/12/31	8.0	ID/2/70b	1.0				
MR/12/32	4.0	ID/2/71	0.25				
LU/12/34	0.25	ID/2/72	4.0				
LU/12/35	4.0	ID/2/73	0.25				
LU/12/37	1.0						
MR/1/38	0.25						
MR/1/39	4.0						
MR/1/40	4.0						
MR/1/41	0.25						
MR/1/42	8.0						
Total		n = 58		n = 9		n = 7	

Key: P.Code= Patients' hospital code number, MR= Medical Research, ID= Infectious Disease hospital and LU= LUTH hospital.

resistance. This study could not ascribe the fluconazole resistance recorded in this study to any particular reason due to paucity of adequate information and non-availability of previous case notes accompanying the patients that participated. History/record of previous exposure was part of the original design but were not obtained because recent knowledge of their HIV sero-status affected their composures and hence made it extremely difficult to volunteer information to this effect.

This result showed that 86.7% of *Candida albicans* and 84.6% of *C. tropicalis* isolated were highly susceptible to fluconazole and that only 7(9.5%) showed invitro resistance. The susceptibility profile of the sensitive candida organisms showed activity at a relatively low MICs (mean MIC 2.2 µg/ml), only 2 strains had MIC of 8.0µg/ml. Although, isolates of *Candida glabrata* has been reported to often generate considerable high fluconazole MICs, with about 15% of isolates being completely resistant rapidly[12]. Lynch[32] reported that *C. glabrata* and *Saccharomyces cerevisae* had several fold increase in MIC of fluconazole tested at 0.31 – 40.0 µg/ml, Pfallen[12] reported 1.25 – 2.5µg/ml, for *C. albicans*, 5.0 – 50 µg/ml, for *C. glabrata*, 0.025 – 0.10 µg/ml, for *C. parapsilosis* and *C. tropicals*, 2.5µg/ml. Also, Carrillo-Munoze[33] reported mean MIC of 5.53µg/ml, for oral Candida isolates. We report relatively lower

Table 4: the fluconazole susceptibility profiles of various yeast species isolatED

Candia Species	No Isolated	Sens. No	(%)	Sens. Dd No	(%)	Resistant No	(%)
C .albicans	30	26	86.7	1	3.3	3	10
C. tropicalis	13	11	84.6	1	7.7	1	7.7
C. krusei	5	2	40.0	1	20.0	2	40
C. glabrata	4	4	100	-		-	
C.pseudotropicalis	3	1	33.3	2	66.7	-	
C.parapsilosis	3	1	100	2	66.7	-	
C. famata	3	3	100	-		-	
C. kefyre	2	2	100	-		-	
C.gulliermondii	1	1	100	-		-	
R. rubra	1	1	100	-		-	
T. cutaneum	1	-	-	1	100		
C. dubliniensis	1	-	-	-			
C. neoformance	4	3	75	1	25	-	
Indeterminate	3	3	100	-		-	
Total No:	74	58	(78.4)	9	(12.1)	7	(9.5)

MICs comparatively which indicate that fluconazole is still efficacious in the management of candidal and non-albicans yeast-like cells infections in HIV and AIDS patients from this locality.

However, that 3 out of the 7 species that screened resistant are *Candida albicans* raise some concern, since at least one person from whom these resistant strains was isolated had well documented referral note and a history of previous exposure, to fluconazole.

This study reports 9.5% fluconazole refractile Candida infection amongst the HIV/AIDS patients in Lagos and is in agreement with the report of 5-10% by Million[13] but Priscilia de Laef Sant' Ana[1] reported up to 6 to 36% in 2002. This may be suggestive of increase in the incidence of resistant strains of OPC probably resulting from repeat use of fluconazole in developed countries compared to the developing setting.

Again, the unconfirmed *Candida dublinensis*, which could have been the first to be reported in Nigeria, could not be confirmed due to non-availability of CHROM agar (Candida chromogenic) medium that is only available medium for identifying colonies of *C. dubliniensis*[20].

Following these findings the primary outcome of the study highlighted the candida/non-albicans species distribution within this setting and demonstrated the existence of fluconazole antifungal resistant yeast species amongst HIV/AIDS patients.

The work equally reviewed that the emergence of resistance occurred following prolonged therapy, and that switching (the ability of Candida species to generate a variety of phenotypes) is a virulent factor[34]. The outcome of this study and those gathered from reviewed articles has lead to our secondary outcome; that is, the need to carryout intermittent if not routine invitro anticandidal susceptibility check, on yeast isolates from HIV/AIDS patients presenting with all kinds of candidiasis, as done for bacteria infections. These recommendations were also supported by other researchers [35] .

Furthermore, with the knowledge that *C. krusei* is intrinsically resistant to fluconazole indicates the need to speciate candida isolates, so as to provide early and specific remedy and avoid mortality. Although routine susceptibility tests and speciation using sugar fermentation and assimilation are both capital intensive, time consuming and strenuous, more rapid methods may be useful.

Finally, this study has indicated the urgent need for further study on HIV/AIDS patients exposed to treatment with antifungal with either failed treatment or repeat episodes, probably, when they are more relaxed and may have overcome the trauma of fresh knowledge of their HIV serostatus.

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