

Species distribution & antifungal susceptibility pattern of oropharyngeal *Candida* isolates from human immunodeficiency virus infected individuals

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Background & objectives: The changing spectrum of *Candida* species in causation of oropharyngeal candidiasis and their antifungal susceptibility pattern among the HIV infected individuals has made the identification to species level mandatory and detection of drug resistance necessary for patient care. The present study was carried out to determine the species distribution and antifungal susceptibility profile of oral *Candida* isolates colonizing or infecting both HIV seropositive and seronegative individuals.

Methods: A case-control study was conducted including 141 consecutive, non-repeat HIV-seropositive individuals and an equal number of sex and age matched HIV-seronegative control. Speciation of the oropharyngeal *Candida* isolates was done using standard yeast identification protocol. Antifungal susceptibility testing was done by the disk-diffusion method as well as by Fungitest method.

Results: From the 59 culture positive HIV seropositive cases, 61 *Candida* isolates were recovered; *Candida albicans* (n=47, 77.0%), *C. dubliniensis* (n=9, 14.7%), *C. parapsilosis* (n=2, 3.2%), *C. glabrata* (n=2, 3.2%), and *C. famata* (n=1, 1.6%). *Candida* colonization in HIV-seropositive individuals was significantly higher than that of HIV-seronegative (control) group. Antifungal susceptibility testing revealed (n=6, 9.3%) *C. albicans* isolates resistant to voriconazole and fluconazole by disk-diffusion method whereas no resistance was seen by Fungitest method.

Interpretation & conclusions: *C. albicans* was the commonest *Candida* species infecting or colonizing HIV seropositive individuals. Oropharyngeal *Candida* isolates had high level susceptibility to all the major antifungals commonly in use. Increased level of immunosuppression in HIV-seropositives and drug resistance of non-albicans *Candida* species makes identification and susceptibility testing of *Candida* species necessary in different geographical areas of the country.

Key words Antifungal susceptibility testing - *Candida* colonization - geographical variation - non-albicans *Candida* species - oropharyngeal candidiasis

Oropharyngeal candidiasis (OPC) is the most frequent opportunistic infection encountered in human immunodeficiency virus (HIV)-infected individuals and is considered as an independent predictor of immunodeficiency in patients with AIDS¹. Though *Candida albicans* is the most frequently isolated species as colonizer and pathogen of the oral mucosa, other *Candida* species, such as *C. tropicalis*, *C. krusei*, *C. parapsilosis* and *C. glabrata* have also been recovered increasingly¹⁻³. The azoles, particularly fluconazole, remain among the most common antifungal drugs, but their intensive clinical use for both therapy and prophylaxis has favoured the emergence of resistant strains³.

Although many studies have been conducted on oropharyngeal candidiasis in HIV population across the country³⁻⁶, there is paucity of such studies in the north-east region of India. Thus the present study was carried out to determine the species distribution and antifungal susceptibility profile of oropharyngeal *Candida* isolates colonizing or infecting both HIV seropositive and seronegative individuals.

Material & Methods

This study was conducted over a period of one year from April, 2011 to March, 2012 in the department of Microbiology, Assam Medical College and Hospital, Dibrugarh, Assam, India. Clearance from the ethical committee of the institute was obtained for the study. A total of 141 consecutive, non-repeat HIV-seropositive individuals, irrespective of oropharyngeal lesions attending Integrated Counselling and Testing Centre (ICTC) either for HIV serostatus detection or for CD4 cell counting were included in the study. The subjects already treated with highly active antiretroviral therapy (HAART) were excluded from the study. The participants were grouped on the basis of age as ≤ 20 , 21-40, 41-60 and >60 yr. Equal number of HIV-negative individuals who came for detection of their HIV serostatus without oral mucosal lesions were included as control group. For each case, an individual of same sex and age falling within the range of that age group was selected as control. An informed written consent was obtained from all adult participants and parents/guardians in case of children.

A standardized data collection form was used to retrieve demographic and personal information and other relevant information like route of HIV transmission, diabetes status, history of tuberculosis, history of drug (antibacterial or antifungal) intake, etc. After

thorough examination of oral cavity, the HIV-positive subjects were further divided into two groups: subjects with oral/oropharyngeal candidiasis (symptomatic seropositive) and subjects without oral/oropharyngeal candidiasis (asymptomatic seropositive). The clinical stages of disease were determined according to the World Health Organization (WHO) guidelines⁷.

Laboratory methods: HIV serostatus of the patients was determined by commercially available ELISA (SD HIV ELISA 3.0, Standard Diagnostic Pvt. Ltd.; Republic of Korea) and three rapid antibody tests using National AIDS Control Organization (NACO) recommended algorithm⁸. CD4 cell counts were measured by using Partec CyFlow counter (Partec GmbH, Munster, Germany).

Two oral swabs were collected from each patient by firmly swabbing the lesion site in case of symptomatic individuals and the dorsum of tongue and buccal mucosa in case of asymptomatic ones. One swab was used to prepare a smear for Gram staining. The second swab was immediately inoculated on two slopes of Sabouraud dextrose agar (SDA) supplemented with antibiotics (50 $\mu\text{g}/\text{ml}$ of chloramphenicol and 5 $\mu\text{g}/\text{ml}$ of gentamicin) and two slopes of SDA without antibiotics. Two sets of tubes were incubated at 37° C and room temperature respectively and examined every 48 h till growth was obtained. Growth of yeast-like organisms was confirmed by Gram staining. Species identification of the isolates was performed by standard methods, i.e. germ tube test, corn meal agar morphology (Dalmau plate technique), carbohydrate fermentation and assimilation (auxanographic) technique, urea hydrolysis and growth at 45°C^{9,10}, colony colour on Hicrome *Candida* differential agar (Hi-media, Mumbai)¹¹, and Vitek 2 identification system (BioMerieux, France). After the final identification, isolates were stored at -80°C in 50 per cent glycerol until susceptibility tests were performed.

In vitro antifungal susceptibility testing: The *in vitro* activities of fluconazole (25 μg), voriconazole (1 μg) (procured from Hi-media, Mumbai) was determined by using disk-diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines for antifungal disk diffusion susceptibility testing of yeasts (M44-A2)¹² and results were expressed as susceptible, susceptible- dose dependent and resistant as per Zone Diameter Interpretive Standards-CLSI¹³. Commercially available modified microtitre broth breakpoint test from BIO-RAD, France (Fungitest™)¹⁴ was also used for susceptibility testing for fluconazole (8-64 $\mu\text{g}/\text{ml}$),

itraconazole (0.5-4 µg/ml), ketoconazole (0.5-4 µg/ml), miconazole (0.5-8 µg/ml), amphotericin-B (2-8 µg/ml) and 5-fluorocytosine (2-32 µg/ml) and interpreted according to the manufacturers' instructions. Results were expressed as no growth or sensitive (isolate inhibited by antifungal agent *in vitro*), low growth (intermediate) and growth or resistant (not inhibited by the antifungal agent *in vitro*).

Quality control: Quality control procedures were performed as per CLSI guidelines¹² using *C. albicans* ATCC 90028 and *C. parapsilosis* ATCC 22019 strains, as quality control strains for fluconazole and voriconazole disk diffusion testing.

Statistical analysis: Data entry, database management and analysis was done using SPSS version 16.0. Student's t test was performed to compare the continuous variable and chi-square test was performed to compare the categorical variables.

Results

The age of HIV-seropositive individuals (n=141) ranged from 2-65 yr with mean age of 35±11.95 yr with female preponderance (male: female ratio was 1:1.1). Ninety three (66.0%) individuals belonged to the age group of 21-40 yr. Among all, 78.7 per cent (n=111) were married and 58.2 per cent (n=82) were rural inhabitants. History of alcohol intake was positive in 31.9 per cent (n=45) cases and 24.8 per cent (n=35) were smoker. Most of the seropositive patients (70.9%, n=100) were categorized into WHO clinical stage 1 followed by 15.6 per cent (n=22), 8.5 (n=12) and 5 per cent (n=7) in clinical stages 3, 2 and 4, respectively. Heterosexual route (83.0%, n=117) was the most common mode of infection followed by blood transfusion (5.7%, n=8), vertical transmission (4.3%, n=6) and homosexual route (2.8%, n=4). One patient was intravenous drug user (needle sharing) while modes of transmission of 3.5 per cent (n=5) individuals were unknown. Among the HIV-seropositives 14 (9.9%) were co-infected with tuberculosis, 15 were hospitalized and 17 individuals had history of antifungal treatment within preceding 90 days. In control group, all individuals were asymptomatic. No obvious risk factors for colonization have been observed.

Out of the 141 HIV seropositive individuals, 27 (19.1%) presented with oropharyngeal lesion (symptomatic seropositive); of whom, 66.7 per cent (n=18) experienced oropharyngeal lesion for the first time while the rest had recurrent episodes. Among them, 63 per cent (n=17) had pseudomembranous lesions

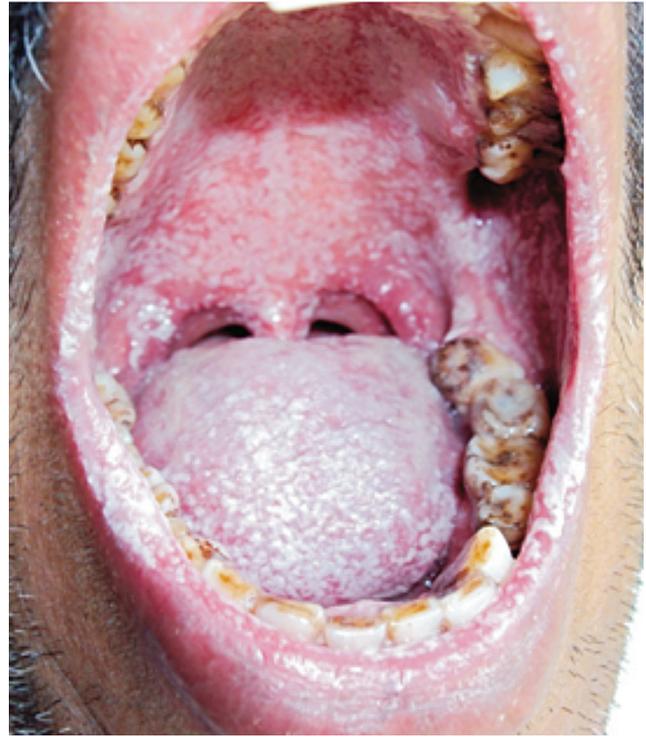


Figure. Oropharyngeal candidiasis-diffuse lesions.

(Figure) while the remaining (n=10) presented with erythematous type. The mean CD4 count in the group with oral/oropharyngeal candidiasis was significantly lower than the group without oral/oropharyngeal candidiasis (117 versus 360 cells/µl) ($P<0.001$).

Of the HIV-seropositive group, a significantly higher number of (41.8%, n=59) individuals were culture positive yielding 61 *Candida* isolates (2 cases yielded mixture of two isolates each) in comparison to the 22.7 per cent (32 isolates) culture positive individuals from control group ($P<0.001$). Isolates were further identified as *C. albicans* (n=47), *C. dubliniensis* (n=9), *C. parapsilosis* (n=2), *C. glabrata* (n=2) and *C. famata* (n=1). *C. dubliniensis* (n=9) was the most common non-albicans *Candida* species isolated from HIV-seropositive group while HIV seronegative (control) group revealed highest of 11 *C. dubliniensis* isolates. Other species isolated from control group were *C. albicans* (n=17), *C. parapsilosis* (n=3) and *C. famata* (n=1). The combinations of mixed isolates were *C. albicans* + *C. glabrata* and *C. albicans* + *C. parapsilosis*. These two cases were excluded from statistical analysis. *C. dubliniensis* isolates were identified by bunch of chlamyospore formation in corn meal agar, dark green colony on HiChrome *Candida*

Differential agar, no xylose assimilation and sparse or no growth at 45°C to differentiate between these two species. The isolation rate of *Candida* species from the HIV-seropositive symptomatic group was 81.5 per cent (n=22) with 72.7 per cent (n=16) *C. albicans* and 27.3 per cent (n=6) non albicans *Candida* isolates. The distribution of *C. albicans* and non-albicans *Candida* isolates between HIV-seropositive and seronegative (control) groups was found to be significant ($P<0.001$) (Table I).

The HIV seropositive asymptomatic group (n=114) revealed *Candida* colonization of 30.7 per cent (n=35) which includes 82.8 per cent (n=29) *C. albicans* and 17.1 per cent (n=6) non albicans *Candida* isolates. HIV seronegative individuals (control group) revealed 22.7 per cent (n=32) colonization of *Candida* species, of which 53.1 per cent (n=17) were *C. albicans* and 46.9 per cent (n=15) non-albicans *Candida* species. *Candida* colonization in HIV-seropositive individuals was significantly ($P<0.05$) higher than that of the HIV seronegative control group.

C. albicans isolates (n=45) were associated with significantly higher mean CD4 cell count of $242 \pm$

207.8 cells/ μ l than 144 ± 119.6 cells/ μ l of non-albicans *Candida* (n=12) isolates ($P<0.05$). *Candida* isolation rate was similar (88.9%) in recurrent cases as well as the cases with first episode of candidiasis.

Factors associated with Candida colonization of asymptomatic HIV-seropositive individuals: Factors found to be significantly associated with candida colonization in HIV seropositive asymptomatic individuals were hospitalization ($P<0.05$), and CD4 cell count of ≤ 200 cells/ μ l ($P<0.001$) (Table II). Factors not associated with increased rate of colonization were male respondents, literacy, employed status, marital status, urban habitation, alcohol consumption, heterosexual mode of HIV transmission and co-infection with tuberculosis.

In univariate logistic regression analysis, factors found to be significantly associated with *Candida* colonization were WHO clinical stage 3, hospitalization and CD4 cell count ≤ 200 cells/ μ l while in multivariate analysis by adjusting the age, only CD4 cell count ≤ 200 cells/ μ l was found to be significant (Table III). Both univariate odds ratio (OR: 4.15, 95% CI, 1.98-8.69; $P<0.01$) and adjusted odds ratio (OR; 5.17, CI 95%, 2.31-11.55; $P<0.01$) of species *C. albicans* were found to be significantly associated with increased risk of HIV seropositivity in conditional logistic regression analysis. Though not significant, the factor non-albicans *Candida* species showed increased risk of HIV seropositivity in both univariate and adjusted regression analysis. Additional analysis among control group showed that the presence of tuberculosis was

Table I. Distribution of *Candida* isolates in HIV seropositive and seronegative groups

Species	HIV negative (n=32)	HIV positive (n=57)
<i>Candida albicans</i>	17 (53.1)	45 (78.9)***
Non-albicans <i>Candida</i> spp.	15 (46.9)	12 (21.1)***

*** $P<0.001$ compared to HIV-negative group

Table II. Characteristics among asymptomatic HIV seropositive individuals (n=114) with *Candida* colonization

Characteristics	Candida colonization		P value
	No (n=78)	Yes (n=36)	
WHO clinical stage			
Stage 1	69 (88.5)	25 (69.4)	0.062 (Fisher's exact test)
Stage 2	3 (3.8)	3 (8.3)	
Stage 3	4 (5.1)	6 (16.7)	
Stage 4	2 (2.6)	2 (5.6)	
Hospitalization			
No	76 (97.4)	31 (86.1)	<0.05 (Fisher's exact test)
Yes	2 (2.6)	5 (13.9)	
CD₄ cell count (cells/μl)			
≤ 200	10 (12.8)	16 (44.4)	<0.001 (Chi square test)
>200	68 (87.2)	20 (55.6)	

Table III. Factor associated with positive *Candida* colonization among asymptomatic HIV seropositive individuals in univariate (crude OR) and multivariate logistic (adjusted OR) regression analysis

Factors	Crude OR ¹	95% CI	Adjusted OR ^{1,†}	95% CI
WHO clinical stage				
Stage 1	Reference		Reference	
Stage 2	2.76	0.52-14.58	1.55	0.24-9.80
Stage 3	4.14*	1.08-15.89	0.72	0.11-4.66
Stage 4	2.76	0.37-20.65	0.63	0.05-8.34
Hospitalization				
No	Reference		Reference	
Yes	6.13*	1.13-33.29	2.27	0.28-18.38
CD ₄ cell count (cells/μl)				
≤200	5.44**	2.14-13.85	4.85*	1.36-17.35
>200	Reference		Reference	

¹Dependent variable: *Candida* colonization (yes/no); [†]adjusted age in multivariate logistic regression analysis; **P*<0.05 & ***P*<0.01; OR: odds ratio

Table IV. Antifungal susceptibility profile of *Candida* isolates by disk diffusion technique

Species	FLU			VOR		
	S	SDD	R	S	SDD	R
<i>Candida albicans</i> (n=64)	58	-	6	58	-	6
<i>C. dubliniensis</i> (n=20)	20	-	-	20	-	-
<i>C. parapsilosis</i> (n=5)	5	-	-	5	-	-
<i>C. glabrata</i> (n=2)	2	-	-	2	-	-
<i>C. famata</i> (n=2)	2	-	-	2	-	-

S, susceptible; R, resistant; SDD, susceptible dose dependant; FLU, fluconazole; VOR, voriconazole

significantly associated with candida colonization (data not shown).

Antifungal susceptibility profile: Antifungal susceptibility testing by disk diffusion method revealed 9.3 per cent (n=6) *C. albicans* isolates to be resistant to each of fluconazole and voriconazole whereas Fungitest method revealed no fluconazole resistance among these isolates except one intermediate to all the azoles (Tables IV, V). Susceptibility to voriconazole could not be compared as Fungitest method lacks this antifungal agent. None of the non-albicans *Candida* isolates showed resistance to fluconazole and voriconazole by disk-diffusion method but some of these isolates were found intermediate to azoles by Fungitest method. All the isolates were found to be susceptible to amphotericin B and 5-fluorocytosine. None of the *Candida* isolates from the HIV-seropositive individuals with previous exposure to

antifungal drugs showed resistance to any antifungal agents tested.

Discussion

The present study showed that oral/oropharyngeal candidiasis was a common manifestation of declining immune system in HIV-seropositive individuals. Mean CD4 cell count of patients with oral/oropharyngeal candidiasis in the present study was 117 cells/μl that correlated well with previous studies from other parts of India¹⁵⁻¹⁷. However, quantitative estimation of yeast carriage and phenotypic changes over time will be a better predictor of level of immunosuppression in HIV seropositive individuals. Finding of pseudomembranous candidiasis as the commonest type of lesion in the present study was consistent with earlier findings^{4,15}.

The colonization was significantly higher in HIV-seropositive group than that of control group as has been

Table V. Antifungal susceptibility profile of *Candida* isolates by Fungitest

Species	Antifungal agent	Fungitest (BIORAD)		
		S	I	R
<i>C. albicans</i> (n=64)	5-FC	64	-	-
	Amp B	64	-	-
	MCZ	63	1	-
	KET	63	1	-
	ITR	63	1	-
	FLU	63	1	-
<i>C. dubliniensis</i> (n=20)	5-FC	20	-	-
	Amp B	20	-	-
	MCZ	20	-	-
	KET	20	-	-
	ITR	19	1	-
	FLU	20	-	-
<i>C. parapsilosis</i> (n=5)	5-FC	5	-	-
	Amp B	5	-	-
	MCZ	5	-	-
	KET	5	-	-
	ITR	5	-	-
<i>C. glabrata</i> (n=2)	5-FC	2	-	-
	Amp B	2	-	-
	MCZ	2	-	-
	KET	-	2	-
	ITR	-	2	-
<i>C. famata</i> (n=2)	5-FC	2	-	-
	Amp B	2	-	-
	MCZ	-	2	-
	KET	2	-	-
	ITR	-	2	-
	FLU	2	-	-

S, susceptible; R, resistant; I, intermediate; 5-FC, 5-fluorocytosine; Amp B, amphotericin B; MCZ, miconazole; KET, ketoconazole; ITR, itraconazole; FLU, fluconazole

reported earlier^{3,16,17}. Our study revealed *C. albicans* as the predominant species in both the seropositive as well as seronegative groups as reported in previous studies^{5,6}. There were some differences in the spectrum of non-albicans *Candida* species and the percentage of recovered isolates observed in studies from different parts of India. A higher number of *C. dubliniensis* isolates in our study indicates a need of further study in this geographical region. Some of the previous studies reported *C. tropicalis* or *C. krusei* as the predominant non-albicans *Candida* species^{5,6} though Nadagir *et al*¹⁸ reported *C. dubliniensis* as the predominant non-albicans species in their study. As *C. dubliniensis* is phenotypically related to *C. albicans*, there may be the possibility of missing this non-albicans species in most of the laboratories. Emergence of *C. dubliniensis* in HIV-seropositive individual is a cause of concern because of its ability to develop drug resistance to commonly used antifungal, fluconazole that may lead to increased morbidity and mortality in HIV-infected individuals with oro-pharyngeal candidiasis^{19,20}. However, none of the *C. dubliniensis* isolates in our study was found to be resistant to fluconazole. Also no azole resistance was found in *C. glabrata* and *C. famata* isolates in the present study which was in contrast to the findings from Mane *et al*³ (50% resistant to azoles).

Our study had some limitations. Molecular methods would have been useful for confirmation of the species identified phenotypically. Further, lesser number of non-albicans *Candida* species isolates limited the scope of this study in predicting their susceptibility pattern in this geographical area.

In conclusion, *C. albicans* was the commonest *Candida* species infecting or colonizing HIV-seropositive individuals and *C. dubliniensis*, the most common non-albicans *Candida* species isolate. In view of increased level of immunosuppression due to HIV seropositivity and drug resistance being more commonly associated with non-albicans *Candida* species; it becomes important to identify the *Candida* up to species level in different geographical areas of the country with their antifungal susceptibility pattern.

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Conflicts of Interest: None.

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