Original Article

Diversity and antifungal resistance patterns of prevalent opportunistic pathogenic yeasts colonizing the oral cavities of asymptomatic human immunodeficiency virus-infected individuals, and their relation to CD4+ counts

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Abstract

Background: Yeasts are important opportunistic pathogens, in individuals infected with human immunodeficiency virus (HIV). Yeast species inhabiting the oral mucosa of HIV-infected persons can act as source of oral lesions, especially as the individual progresses towards immunocompromised state. Present study was conducted to evaluate the diversity of yeasts in oral cavities of asymptomatic HIV-infected persons and their association with CD4+ cell counts. **Materials and Methods:** 100 HIV seropositive subjects and 100 healthy controls were screened for oral yeast carriage using standard procedures. **Results:** Of the 100 HIV-seropositive persons screened, 48 were colonized by different yeasts, either alone or in association with another species. *Candida albicans* was the most common species (56.90%) while non *C. albicans Candida* (NCAC) accounted for 39.65%. Among NCAC, *Candida tropicalis* and *Candida krusei* were most common. One isolate each of rare opportunistic pathogenic yeasts, *Geotrichum candidum* and *Saccharomyces cereviseae,* was recovered. The control group had an oral candidal carriage rate of 23%; *C. albicans* was the predominant species, followed by *Candida glabrata*, *C. tropicalis* and *Candida parapsilosis.* Antifungal susceptibility testing revealed no resistance in *C. albicans,* to the commonly used antifungal agents, whereas resistance or dose dependent susceptibility to fluconazole was observed in some of the NCAC species. **Conclusion:** Oral carriage of opportunistic pathogenic yeasts was greater in HIV-seropositive persons heading towards immunocompromised state, as evidenced by their CD4+ cell count. The predominant yeast isolated in this study (*C. albicans*), was found to be susceptible to commonly used antifungals.

Key words: Antifungal susceptibility test, CD4+ count, human immunodeficiency virus, seropositive, opportunistic infections, oral candidiasis, oral yeast carriage

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INTRODUCTION

The oral cavity of human beings is rich in microbial flora of extremely diverse nature, with opportunistic pathogenic yeasts being important components.[1] These yeasts are often a source of local infection, especially in immunocompromised patients, as

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in human immunodeficiency virus (HIV)/AIDS disease.[2] India is home to a large number of people living with HIV/AIDS, where many do not have access to antiretroviral therapy (ART), and oropharyngeal candidiasis (OPC) has been reported as the most common HIV-associated opportunistic infection.[2] Among the yeasts inhabiting the oral cavity, genus *Candida*, specifically the most virulent species, *Candida albicans*, is found to be commonly distributed either as colonizer or as pathogen.[3-6] Furthermore, there are other yeasts, previously thought to be nonpathogenic, now gaining importance, especially in the era of HIV/AIDS.[7,8] In the Indian scenario, only a few reports are available on asymptomatic carriage of yeasts in the oral cavity of HIV seropositive individuals.[3-5,9,10] Most of these studies are, however, from the southern states of the country.[3,4,9] Of these, still fewer studies have looked for the association of yeast carriage in relation to $CD4^+$ count.[5,9]

Present study was planned to determine the current status of opportunistic yeasts, especially *Candida*, including non *C. albicans Candida* (NCAC), in the oral cavities of asymptomatic HIV seropositive persons. The study also explores the antifungal susceptibility patterns of the prevalent isolates.

MATERIALS AND METHODS

Oral swabs were collected from consecutive HIV seropositive individuals, attending ART center of Safdarjung Hospital, New Delhi, on the particular day of sample collection. Control group included 100 healthy volunteers from staff and student population of Safdarjung hospital, who were age and sex matched. None of these subjects had any underlying predisposing conditions, as explored and ruled out by a detailed questionnaire. Subjects excluded were those with a history of treatment with antifungals during previous 6 months, with dentures, with pregnancy or on oral contraceptives. Samples from the oral mucosa were collected from both male and female HIV-infected persons (68 + 32 = 100) (Confirmed by 3 rapid tests), attending the ART center. None of the subjects had any clinical signs of oral lesions at the time of sample collection. A detailed history-taking preceded the collection of samples. $CD4^+$ lymphocyte count was performed on the same day as collection of oral samples. Informed consent was obtained from each subject. The study was approved by Institutional Ethics Committee (No. 26-11-EC [21/31]).

Two swabs were collected by depressing the tongue and gently rubbing the surface of gum, tooth, tonsils and tongue using sterile cotton swabs. The first swab was inoculated on Sabouraud's Dextrose Agar (SDA) and incubated at 37°C for up to 7 days, and observed daily for growth, while the second swab was used to prepare a smear for Gram staining. All isolates were identified by their morphology on Gram's smear, cultural characteristics on SDA, Corn Meal Agar with 1% Tween 80, Chrom Agar (color, texture, etc.), germ tube test and carbohydrate assimilation/fermentation tests.[11] When conventional methods failed to identify unusual isolates, they were verified by automated Vitek-2 YST system. To differentiate *C. albicans* from *Candida dubliniensis*, all *C. albicans* isolates (confirmed by conventional methods) were subjected to growth at 45°C and Tween 80 hydrolysis test, along with the ATCC control strains.[12] Subsequent to species identification, antifungal susceptibility testing was performed, using fluconazole (FL), ketoconazole, voriconazole and amphotericin B, by *E*-test strip method. This was performed by suspending a portion of the isolated colony in normal saline and adjusting its turbidity to 0.5 McFarland standard. The suspension was then spread over the surface of a predried RPMI-1640 +2% glucose agar media, using a sterile cotton swab. *E*-test strips were applied using a sterile forceps and minimum inhibitory concentrations (MICs) were determined after 24 h and 48 h of incubation at 37°C.[13] In the case of doubt in the results by *E*-test, MIC was confirmed by micro broth dilution method, to determine the discordance if any. MIC for each antifungal agent was interpreted as per Clinical and Laboratory Standards Institute criteria.[14] $CD4^+$ cells were analyzed and enumerated on a FACS count flow cytometer (Becton Dickinson, California, USA), using two color monoclonal antibody panel.

Data analysis

Data analyses were performed by Chi-square test using GraphPad prism software version 5.00 developed by GraphPad Software, Inc., La Jolla, CA, USA. A $P < 0.05$ was considered significant.

RESULTS

Of the 100 HIV-seropositive persons investigated, 48 (48.00%) were colonized with different species of yeasts, while 52 (52.00%) had a negative culture. While 39 specimens yielded single species, nine yielded more than one species, giving a total of 58 isolates. Of the 58 isolates, from HIV seropositive individuals, *C. albicans* was the most frequently isolated species, 33 (56.90%), followed by an equal distribution (15.52%) of *Candida tropicalis* and *Candida krusei* [Table 1]. Of the nine specimens,

yielding multiple isolates, six had a combination of *C. albicans* with various NCAC, while three had a combination of two NCAC species (*C. tropicalis* and *C. krusei*, *C. tropicalis* and *Candida glabrata* and *C. glabrata* and *C. krusei)*. Of the 100 samples from healthy controls, only 23 yielded a growth of *Candida*, with a predominance of *C. albicans* (18), followed by *C. glabrata* (3), *Candida parapsilosis* (1) and *C. tropicalis* (1) [Table 1].

Relationship between yeast carriage and the CD4$^+$ count

According to CD4$^+$ count, HIV seropositive subjects were classified into three groups: Group A: CD4$^+$ count >400 cells/μl; Group B: CD4$^+$ count between 201 and 400 cells/μl; Group C: CD4$^+$ count <200 cells/μl.

Yeasts isolated from Group A

A total of 30 samples were collected from the subjects belonging to Group A. Of these, eight (26.66%) yielded different yeast species [Table 2], six of which grew only single species, two yielded multiple species, giving a total of 11 isolates [Table 1]. A rare opportunistic pathogenic yeast *Saccharomyces cereviseae* was isolated in combination with *C. albicans* and *C. krusei* from this group. Besides biological activities (seen by Vitek-2 YST system), identification of *S. cereviseae* was also confirmed by genetic study (from a reference center, PGIMER, Chandigarh).

Table 1: Species distribution of various yeasts colonizing the oral cavities of HIV seropositive individuals and healthy controls

Species	HIV+ (100) Number of isolates				Control (100)
	Group A	Group B	Group C	Total	
Candida albicans	4	17	12	33	18
Candida tropicalis	3	3	3	09	1
Candida krusei	3	2	4	09	0
Candida glabrata	0	2	2	04	3
Candida parapsilosis	0	0	1	01	1
Saccharomyces cereviseae	1	0	0	01	0
Geotrichum candidum	0	0	1	01	0
Total	11	24	23	58	23

Group A: CD4$^+$ count >400 cells/µl; Group B: CD4$^+$ count between 200-400 cells/µl; Group C: CD4$^+$ count <200 cells/µl.
HIV=Human immunodeficiency virus

Table 2: Oral yeast carriage in relation with CD4$^+$ cell count

CD4$^+$ cell count (cells/µl)	Total number of subjects	Number of subjects yielded growth (%)	*P*
>400	30	8 (26.67)	*P*=0.003
200-400	42	20 (47.61)	
<200	28	20 (71.43)	

Yeasts isolated from Group B

Of 42 samples collected from the subjects belonging to Group B, 20 (47.62%) yielded different *Candida* species [Table 2]. While 16 of these grew only one single species, four yielded multiple species, giving a total of 24 isolates [Table 1].

Yeasts isolated from Group C

A total of 28 samples were collected from subjects belonging to Group C. Of these; 20 (71.43%) yielded yeast isolates [Table 2]. 17 of which grew only single species, including an unusual yeast *Geotrichum candidum*. Three specimens yielded multiple species, giving a total of 23 isolates [Table 1].

Antifungal resistance patterns of the isolates

In vitro antifungal susceptibility testing of the isolates revealed that all *C. albicans* isolated from both the control and the HIV-seropositive group did not show resistance to any of the antifungal agents tested. NCAC isolated from the control group also yielded similar findings. Among the HIV-seropositive group, FL resistance was observed in 100% of *C. krusei* isolates and dose dependent susceptibility in two of the four isolates of *C. glabrata* [Table 3].

DISCUSSION

Various species of yeasts inhabit the oral cavity of humans as harmless commensals.[1] However, in recent past, especially in the era of HIV promoted diseases, these yeasts have gained importance as opportunistic pathogens, causing oral lesions.[1] It is also shown that OPC, is the most common opportunistic infection in HIV-seropositive individuals, as the disease progresses.[2,3,9] Often, the yeast routinely seen as normal flora are the sources of OPC, as the individual progress toward an immunocompromised state.[1,2]

Earlier studies conducted in India and elsewhere revealed that asymptomatic oropharyngeal colonization with yeast is known to be significantly higher among HIV-infected persons. Published reports shows carriage rates to vary between 27.9% and 68% in this group.[4,9,10] Present study recorded an overall yeast carriage rate of 48%, which included different *Candida* species along with other yeasts. This figure is comparable to those reported by Pavithra *et al.*,[4] from Southern India and Hung *et al.*,[15] from Taiwan, but lesser than that of a similar study carried out a decade ago by Gugnani *et al.*[10] (68% - included

Table 3: Antifungal resistance patterns of the yeast isolates

Species	Amphotericin B	Ketoconazole	Fluconazole	Voriconazole
Candida albicans (*n*=51)				
HIV: 33	S - 33 R - 0	S - 33 R - 0	S - 33 R - 0	S - 33 R - 0
Control: 18	S - 18 R - 0	S - 18 R - 0	S - 18 R - 0	S - 18 R - 0
Candida tropicalis (*n*=10)				
HIV: 9	S - 9 R - 0	S - 9 R - 0	S - 9 R - 0	S - 9 R - 0
Control: 1	S - 1 R - 0	S - 1 R - 0	S - 1 R - 0	S - 1 R - 0
Candida krusei (*n*=9)				
HIV: 9	S - 9 R - 0	S - 9 R - 0	S - 0 R - 9	S - 9 R - 0
Control: 0	-	-	-	-
Candida glabrata (*n*=7)				
HIV: 4	S - 4 R - 0	S - 4 R - 0	S - 2 SDD - 2 R - 0	S - 4 R - 0
Control: 3	S - 3 R - 0	S - 3 R - 0	S - 3 R - 0	S - 3 R - 0
Candida parapsilosis (*n*=2)				
HIV: 1	S - 1 R - 0	S - 1 R - 0	S - 1 R - 0	S - 1 R - 0
Control: 1	S - 1 R - 0	S - 1 R - 0	S - 1 R - 0	S - 1 R - 0

Amphotericin B: S≤0.5 μg/ml, R≥1 μg/ml; Fluconazole: S≤8 μg/ml, SDD 16-32 μg/ml, R≥64 μg/ml; Ketaconazole: R≥1 μg/ml; Itraconazole: S≤0.125 μg/ml, SDD 0.25-0.5, R≥1 μg/ml; Voriconazole: S≤1. S=Sensitive; R=Resistant; SDD=Susceptible dose dependent; HIV=Human immunodeficiency virus

65.35% *Candida* and 2.7% other yeasts) from New Delhi. A recent study by Maurya *et al.*,[5] reported a carriage rate of 33.1% in a similar group of patients.[5] This variance in the isolation rates may be due to the difference in distribution of *Candida* in several geographical areas, time of sampling or the use of different collection methods for yeast recovery.[5,16] Among the control group, candidal carriage was observed in 23% of subjects, which is in agreement with other Indian studies.[4,5]

Interestingly, among the yeasts isolated, the predominant species was *C. albicans* (56.90%), which is in concordance with other reports.[3-5] NCAC species accounted for 39.65%, of which *C. tropicalis* and *C. krusei* head the list. Though the isolation of *C. tropicalis*, as the second most common species, is in agreement with most other Indian reports on asymptomatic carriage or in OPC,[3,4] the scenario is different as far as *C. krusei* isolation is concerned. Only one report from southern India recorded *C. krusei* (26.31%) as the predominant NCAC species isolated from a similar patient group, as in the present study.[4] This spectrum is quite different from the results of a study conducted a decade ago, by one of the authors (UB) on OPC in HIV patients, where not a single isolate was *C. tropicalis*.[2] Another important observation in the present study is the isolation of potentially less opportunistic pathogenic yeasts like *S. cereviseae* and *G. candidum*. *S. cereviseae* was isolated in combination with *C. albicans* and *C. krusei* from one subject. *S. cereviseae*, also known as brewer's yeast, is commonly used in baking and fermentation of alcoholic drinks.[17] It is often considered as a commensal organism of the oral cavity and has been isolated from immunocompromised patients, as a colonizer.[7] Sporadic reports are available in the literature, revealing its presence as a pathogen causing oral lesion and mimicking candidiasis.[18] From India, there is a single report available from New Delhi, on pan gastrointestinal-mucosal involvement, by *S. cereviseae* in an AIDS patient.[19]

Geotrichum candidum was present as a single isolate from another subject. Though rare, various *Geotrichum* species are found to affect the respiratory tract, skin, oral cavity and also invasive infections, in the immunocompromised host.[8] Probably, this is the first report on the isolation of *G. candidum* from Indian HIV seropositive individuals.

The present study has brought forth an important finding, that the yield of potentially pathogenic yeast isolates was highest in the subjects belonging to Group C, with $CD4^+$ counts below 200 cells/μl.

The isolation was lesser in Group B subjects with $CD4^+$ counts between 200 and 400 cells/μl, and least in subjects belonging to Group A, with $CD4^+$ counts >400 cells/μl [Table 2]. This clearly indicates that the carriage of potentially pathogenic yeasts in the oral cavities is greater in patients, as they head toward immunosuppressive state. Though this finding is in corroboration with Maurya *et al.*,[5] there is a difference in observation as reported by Costa *et al.*,[6] and Ananthalakshmi *et al*.[9]

There are reports on emerging FL, a common antifungal used in therapy, resistant strains of *C. albicans* both in HIV seropositive[20] and seronegative persons.[21] Hence, an *in vitro* antifungal susceptibility test of *Candida* species was performed to determine their resistance patterns, and highlight an emerging trend if any, for future reference. Besides as therapy, in HIV seropositive individuals, FL is also recommended in lifelong prophylaxis to prevent complicated severe opportunistic infections in AIDS.[2] Though most Indian studies on *in vitro* antifungal resistance patterns of *Candida* isolates, revealed either nil or very low percentage of FL resistance in *C. albicans* isolated from various clinical specimens,[2] a higher percentage of *C. albicans*, isolated from Intensive Care Unit patients, were found to be resistant to FL.[21]

Interestingly, none of the *C. albicans* isolated from either HIV seropositive or control groups, showed resistance to FL and other antifungals. Absence of drug resistance in these isolates can be attributed to several factors including, degree of prior exposure to a particular drug, acquisition of resistance gene, and the intrinsic lack of resistance of *Candida* species.[2] Although a few HIV seropositive patients were on ART, none of them, was on FL prophylaxis, which probably explains the outcome of *in vitro* susceptibility test results.

The spectrum of results was different in NCAC isolates. Among the isolates from HIV group, resistance to FL was observed in all isolates of *C. krusei* while two isolates of *C. glabrata* showed dose-dependent susceptibility. However, NCAC species from healthy controls did not show resistance to any of the antifungal agents tested [Table 3]. The resistant isolates can be described as primarily or intrinsically resistant strains. Moreover, like *C. albicans*, none of the isolates showed resistance to other antifungal agents tested. However, this is only a 1 time testing; the study should ideally be repeated with isolates from the same patients at different time intervals to observe any changes in the patterns of carriage and susceptibility to various antifungal agents.[2]

To conclude, periodic surveys of oropharyngeal yeast colonization in a high-risk population, such as HIV-infected subjects, are useful in the clinical arena to bring out changes in spectrum if any. This study showcases the present scenario of the yeast distribution and resistance patterns of predominant species, colonizing the oral cavities of HIV seropositive individuals. This will be of help in understanding the current status of distribution of yeast species, in this high-risk patient population, as oral carriage may predispose to the development of clinically overt oral lesions especially in individuals heading toward immunocompromised state. Furthermore, our findings clearly indicate that the carriage of potentially pathogenic yeasts in the oral cavities is associated with reduced $CD4^+$ count.

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