

ORIGINAL ARTICLE

An *in vitro* study of antifungal drug susceptibility of *Candida* species isolated from human immunodeficiency virus seropositive and human immunodeficiency virus seronegative individuals in Lucknow population Uttar Pradesh

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ABSTRACT

Background: Candidiasis is the most common opportunistic infection in human immunodeficiency virus (HIV) seropositive patients, starting from asymptomatic colonization to pathogenic forms and gradual colonization of non-*albicans* in patients with advanced immunosuppression leads to resistance for azole group of antifungal drugs with high rate of morbidity and mortality. **Objectives:** To isolate the *Candida* species and determine of antifungal drug susceptibility against fluconazole, itraconazole, nystatin, amphotericin B, and clotrimazole in HIV seropositive and control individuals, with or without clinical oropharyngeal candidiasis (OPC). **Materials and Methods:** Includes samples from faucial region of 70 subjects with and without clinical candidiasis in HIV seropositive and controls were aseptically inoculated onto Sabaroud's Dextrose Agar media and yeasts were identified for the specific species by Corn Meal Agar, sugar fermentation and heat tolerance tests. Antifungal drug susceptibility of the isolated species was done against above-mentioned drugs by E-test and disc diffusion method. **Results:** The commonly isolated species in HIV seropositive and controls were *Candida albicans*, *Candida glabrata* and *Candida tropicalis* *Candida guilliermondii* and *Candida dubliniensis* isolated only in HIV seropositive patients. Susceptibility against selected antifungal drugs was observed more in HIV-negative individuals whereas susceptible dose-dependent and resistance were predominant in HIV-positive patients. **Conclusion:** Resistance is the major problem in the therapy of OPC, especially in HIV seropositive patients due to aggressive and prolonged use of antifungal agents, therefore, our study emphasizes the need for antifungal drug susceptibility testing whenever antifungal treatment is desired, especially in HIV-infected subjects.

Key words: Antifungal susceptibility test, *Candida*, *Candida dubliniensis*, *Candida guilliermondii*, Corn Meal Agar

INTRODUCTION

Oral cavities are colonized by *Candida albicans* or other yeast species in 40–60% of healthy persons. In the

presence of any local or general predisposing factors, *Candida* may cause acute or chronic oral infections such as

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Figure 1: Human immunodeficiency virus seropositive patient with evidence of oropharyngeal candidiasis



Figure 2: Colonies of *Candida* species

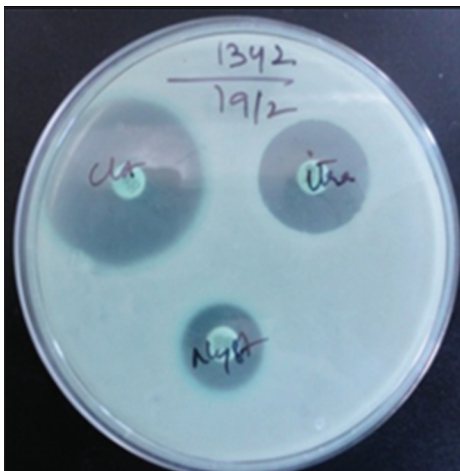


Figure 3: *Candida* species showing zone of inhibition for clotrimazole, itraconazole and nystatin



Figure 4: *Candida* colonies showing susceptibility to fluconazole minimum inhibitory concentration strip



Figure 5: Amphotericin minimum inhibitory concentration strip showing resistance to *Candida* colonies



Figure 6: *Candida* colonies showing resistance to all selected antifungal drugs

pseudomembranous (oral thrush), atrophic (erythematous), angular cheilitis or hyperplastic candidiasis.^[1] The advent of

human immunodeficiency virus (HIV) infection has entirely altered the incidence and prevalence of fungal infections. In HIV/acquired immune deficiency syndrome (AIDS) patients,

oropharyngeal candidiasis (OPC) is the most commonly reported opportunistic infection observed, accounting in approximately 80–95% of those with HIV patients [Figure 1]. The prevalence of OPC in HIV-positive patients appears to be correlated to the severity of the immunological dysfunction. The occurrence of oral candidiasis at initial stages of HIV-infected and AIDS (HIV/AIDS) patients particularly the risk is considered to be higher in patients with a CD4⁺ cell count is low (400–700 cells/mm³) or <200 cells/mm³ and high plasma HIV-RNA loads.^[2] In India, OPC is the second most common opportunistic infection among patients infected with HIV and occurs in more than 95% of AIDS patients and it is considered as an important marker of the AIDS disease and its progression.^[3]

Among various species of *Candida*, *C. albicans* is the main cause of oral candidiasis in HIV infection and AIDS, but in advanced stage, rise and epidemiological shift in candidiasis has resulted in infections with *Candida* species other than *C. albicans*. *Candida dubliniensis* has also been linked to OPC in such patients. Hence, identification of various members of *Candida* species causing infection in HIV patients has gained importance.^[4] Fluconazole is considered to be the drug of choice for the treatment of the most common HIV-associated opportunistic yeast infections. It has been the most widely used drug because of its good absorption, low toxicity and ability to be administered through both oral and intravenous routes and its resistance is associated with prolonged exposure to azoles.^[2]

The common use of fluconazole and other azole antifungal agents for treating this infection has been associated with the emergence of azole-resistant isolates of *C. albicans* in HIV/AIDS patients.^[2] The prolonged management of OPC might cause the development of drug-resistant OPC and there have been reports of the emergence of resistance to antifungal agents in HIV/AIDS patients with OPC.^[5] The increasing resistance to antifungal treatments and expanding drug therapy options has prompted the need for clinically relevant antifungal susceptibility testing (AST).^[6] The aim of this study is to isolate the *Candida* species in HIV-positive and control individuals, with or without clinical OPC and to determine the antifungal drug susceptibility of *Candida* for fluconazole, itraconazole, nystatin, amphotericin B and clotrimazole.

MATERIALS AND METHODS

Study sample size

The prospective case-control study included 70 individuals, who were divided into two studies and two control groups (HIV seropositive = 36 and HIV-seronegative = 34) with and without clinical evidence of oral candidiasis. All of them were tested for HIV by tests (COOMB AIDS, TRI-DOT), according to National AIDS Control Organization guidelines

and confirmed by Western Blot. The protocol for the present study was approved by the Institutional Ethics Committee.

Sample collection

The study sample was collected from both the HIV seropositive and negative patients after thorough clinical examination with their written and verbal consent. The patient was asked to swish the mouth with normal saline to remove superficial debris, with the help of sterile cotton swabs sample was collected from the faucial region by rolling the swab several times across the surface. Immediately, after sampling swab replaced into its sterile container and taken to the laboratory within 2 h. Oral specimens were coded according to clinical group and patient.

Laboratory methods

The collected oral swabs were inoculated onto Sabraud's Dextrose Agar (SDA) supplemented with chloramphenicol, and incubated at 36°C (±1°C) for 2 days and for a 7 days additional period at 30°C (±1°C) before being discharged as negative. The presence of *Candida* was confirmed by the presence of creamy white colonies [Figure 2] and for reconfirmation positive isolates were then analyzed by light microscopy on lactophenol cotton blue wet mount slides to reveal presence of ovoid yeasts (hyphae and pseudohyphae). Yeasts were identified to the species level by standard methods, microscopic morphology on Corn Meal Agar-Tween 80 (pseudohyphae and true hyphae formation, as well as chlamydoconidia production) and sugar fermentation tests (glucose, maltose, sucrose and lactose). In addition, for presumptive identification of *C. dubliniensis*, all isolates identified as *C. albicans* were also tested for growth at 45°C (±1°C) for 48 h on SDA. Isolates were stored in sterile containers for further use. The antifungal agents tested were: Fluconazole, amphotericin B, nystatin, clotrimazole and itraconazole. The isolates were subcultured on SDA and incubated at 37°C overnight. Saline suspensions of isolates were made and the turbidity was adjusted at 0.5 McFarland standards. A lawn culture was done on freshly prepared Muller Hinton Agar (MHA) (Hi-Media) plates. The antifungal E-strips and disks were placed on MHA plates and incubated at 37°C. The zone of inhibition was measured after 24–48 h. The isolates were classified as susceptible, susceptible dose-dependent (SDD) and resistant and interpretive criteria used were according to Clinical and Laboratory Standards Institute guidelines [Figure 3, Table 1].

RESULTS

The present study evaluates the drug susceptibility test in *Candida* isolated from HIV seropositive and HIV seronegative patients in Lucknow population. A total of 70 patients (females = 24 and males = 46) aged between 19 and 69 years were recruited. Of total, 36 (51.4%) were HIV seropositive and 34 (48.6%) were HIV seronegative. Table 2

Table 1: CLSI guidelines for antifungal agents

	Susceptible	Susceptible dose dependent	Resistance
MIC strips (µg/ml)			
Fluconazole	≤8	8-64	≥64
Amphotericin B	≤1	1-4	≥4
Diffusion disc (mm)			
Nystatin	≥15	14-10	<10
Clotrimazole	≥20	19-10	<10
Itraconazole	≥23	22-14	<13

MIC: Minimum inhibitory concentration, CLSI: Clinical and Laboratory Standards Institute

Table 2: Association of HIV seropositivity with demographic and clinical characteristics

Characteristics	HIV seropositive (n=36) (%)	HIV seronegative (n=34) (%)	χ^2/U	P
Age (years)				
15-25	4 (11.1)	4 (11.8)	10.74	0.030
26-35	14 (38.9)	10 (29.4)		
36-45	16 (44.2)	8 (23.5)		
46-55	2 (5.6)	9 (26.5)		
≥56	0 (0.0)	3 (8.8)		
Sex				
Females	15 (41.7)	9 (26.5)	1.79	0.181
Males	21 (58.3)	25 (73.5)		
Oral candidiasis				
Absent	19 (52.8)	20 (58.8)	0.26	0.611
Present	17 (47.2)	14 (41.2)		
Candidiasis type				
Erythematous	2 (11.8)	0 (0.0)	2.44	0.295
Hyperplastic	4 (23.5)	2 (14.3)		
Pseudomembranous	11 (64.7)	12 (85.7)		
Candida species				
Albicans	24 (66.7)	25 (73.5)	13.82	0.008
Dubliniensis	1 (2.8)	0 (0.0)		
Glabrata	9 (25.0)	1 (2.9)		
Guilliermondii	1 (2.8)	0 (0.0)		
Tropicalis	1 (2.8)	8 (23.5)		

shows that HIV seropositivity also not associated ($P > 0.05$) with type of candidiasis though pseudomembranous (64.7%) was comparatively higher than both erythematous (11.8%) and hyperplastic (23.5%) ($P = 0.295$). Table 2 also shows that HIV seropositivity significantly associated ($P < 0.01$) with type of *Candida* species, especially *Candida glabrata* ($P = 0.008$). One isolate of *Candida guilliermondii* and *C. dubliniensis* was also isolated in HIV seropositive patients. The assessment of antifungal drug sensitivity of selected five drugs: Fluconazole, nystatin, amphotericin B, clotrimazole and itraconazole against candidiasis in both HIV seropositive and HIV seronegative patients was done, [Table 3], HIV patients 86.1% were susceptible to fluconazole, whereas

13.9% were dose-dependent and none were resistant. Among the seronegative patients, the susceptibility, dose dependence and resistance to fluconazole were 94.1%, 2.9% and 2.9% respectively [Figure 4]. Against amphotericin B in HIV seropositive patients 66.7% were susceptible, with 22.2% resistant [Figure 5] and rest were 11.1% dose-dependent compared to HIV-seronegative individuals showing 85.3%, 5.9% and 8.8% susceptible, susceptibility dose dependent and resistance, against Nystatin in HIV seropositive 61.1%, 36.1% and 2.8% were SDD and resistant respectively while as in HIV seronegative 91.2%, 8.8%, 0.0% were SDD and resistant, respectively. In HIV-seropositive against clotrimazole, out of 36 isolates 66.7% were (S), 25% (SDD) and 8.3% (R) but in HIV seronegative out of 34 isolates 32 (94.1%) were (S), 2 (5.9%) were (SDD) and none of the isolate was resistant, and on comparison of itraconazole susceptibility between HIV-positive and HIV-negative patients and it was found that susceptibility was higher in HIV-seronegative patients (88.2%) than HIV seropositive patient (52.8%), whereas SDD and resistance was found more in HIV-positive patients [Figure 6]

DISCUSSION

The incidence of OPC has increased over the last several decades due to the widespread use of antibiotics and immunosuppressive drugs to combat conditions associated with immunosuppression such as patients with cancer, HIV/AIDS. Despite some effective treatment options such as azoles and polyenes, OPC is associated with high morbidity and mortality rates.^[2]

The prolonged management of OPC might cause the development of drug-resistant OPC and there have been reports of the emergence of resistance to antifungal agents in HIV/AIDS patients with OPC.^[6] The increasing resistance to antifungal treatments and expanding drug therapy options has prompted the need for clinically relevant AST.^[1]

Among the clinical forms of OPC pseudomembranous was predominant followed by erythematous and hyperplastic candidiasis in HIV seropositive patients.^[7-9] These findings were in contrast with the reported findings^[10,9,11] where erythematous form outnumbered pseudomembranous candidiasis reasoning that patients under antimicrobial therapy and habit of smoking showed increased the incidence of erythematous candidiasis. In the present study, most subjects selected were not on antimicrobials and were nonsmokers. 85.7% of HIV-seronegative individuals showed pseudomembranous candidiasis in the present study.

C. albicans being the normal commensals of the oral cavity is isolated more commonly in OPC of HIV seropositive and HIV seronegative patients due to increased level of immune-suppression that has led the opportunity for their growth and shift to non-*albicans*.^[12-20]

Table 3: Association of HIV seropositivity with antifungal drug susceptibility

Characteristics	HIV seropositive (n=36) (%)	HIV seronegative (n=34) (%)	χ^2	P
Fluconazole				
Susceptible	31 (86.1)	32 (94.1)	3.63	0.163
SDD	5 (13.9)	1 (2.9)		
Resistant	0 (0.0)	1 (2.9)		
Amphotericin B				
Susceptible	24 (66.7)	29 (85.3)	3.36	0.187
SDD	4 (11.1)	2 (5.9)		
Resistant	8 (22.2)	3 (8.8)		
Nystatin				
Susceptible	22 (61.1)	31 (91.2)	8.73	0.013
SDD	13 (36.1)	3 (8.8)		
Resistant	1 (2.8)	0 (0.0)		
Clotrimazole				
Susceptible	24 (66.7)	32 (94.1)	8.55	0.014
SDD	9 (25.0)	2 (5.9)		
Resistant	3 (8.3)	0 (0.0)		
Itraconazole				
Susceptible	19 (52.8)	30 (88.2)	10.75	0.005
SDD	15 (41.7)	3 (8.8)		
Resistant	2 (5.6)	1 (2.9)		

SDD: Susceptible dose dependent

Among non-*albicans* *C. glabrata* and *Candida tropicalis* were isolated predominantly in HIV seropositive and HIV seronegative patients, respectively.

The present study also reported a rare, enigmatic more pathogenic non-*albicans*, *C. dubliniensis* and *C. guilliermondii* in HIV seropositive patients. One strain of *C. dubliniensis* has also been isolated by other authors in different parts of world.^[21-25] *C. guilliermondii* has also been isolated in HIV seropositive patients by other authors.^[17,18,21] In the present study, among non-*albicans* *C. glabrata*, *C. guilliermondii* and *C. dubliniensis* were significantly (0.001) increased in HIV-positive patients whereas *C. tropicalis* was predominant in HIV seronegative patients.

Most predominant species isolated in pseudomembranous candidiasis was *C. albicans* followed *C. glabrata*. In four cases of hyperplastic candidiasis two cases showed *C. albicans* species and two cases showed *C. glabrata*, whereas in two cases erythematous candidiasis one case showed species of *C. glabrata* and one species of *C. guilliermondii*.

The assessment of susceptibility, susceptibility dose-dependent, and resistance of fluconazole against *Candida* species showed higher rate of susceptibility in HIV seronegative patients, whereas dose-dependent was seen higher in HIV-seropositive patients and only one patient was resistant in HIV seronegative patients.^[26,27]

The susceptibility rate for amphotericin B was higher in HIV-seronegative patients while as susceptibility dose-dependent and resistance was seen more in HIV seropositive patients.

The susceptibility rate for nystatin in HIV seronegative patients was much higher as compared to HIV seropositive patients on contrary the susceptibility dose-dependent and resistance rate was more in HIV seropositive patients as compared to seronegative patients and these results were in accordance with the findings of Gutiérrez *et al.* 2002.^[25]

The susceptibility rate to clotrimazole in HIV seropositive patients were less than HIV-seronegative patients whereas dose-dependent and resistant were seen more predominant in HIV seropositive patients, these findings were in accordance with the findings of Gutiérrez *et al.* 2002.^[25]

The susceptibility, susceptibility dose-dependent and resistance rate of *C. albicans*^[23] in HIV seropositive patients against the selected antifungal drugs showed the highest percentage of susceptibility for fluconazole followed by amphotericin B, clotrimazole, nystatin and least susceptibility was found to itraconazole. The SDD was highest against itraconazole and least for amphotericin B. Resistance was found highest to amphotericin B and no resistance was found for fluconazole [Table 4]. Hamza *et al.* 2008^[28] also reported similar findings of no resistance of *C. albicans* to fluconazole but the reported findings by Jeddy *et al.* 2011^[7] and Badiie *et al.* 2010^[21] were in contrast to our findings who found resistance of *C. albicans* to fluconazole in HIV-positive patients. Sánchez-Vargas *et al.* 2005,^[6] Jeddy *et al.* 2011^[7] also reported no resistance to amphotericin B, but the findings of Bignaut *et al.*^[29] 2002, Wabe *et al.* 2011,^[30] Mulu *et al.*^[31] were in accordance with our findings who also reported resistance to amphotericin B.

The species *C. glabrata*^[12] in HIV seropositive patients showed the highest percentage of susceptibility for fluconazole followed by amphotericin B nystatin and clotrimazole and least susceptibility was found to itraconazole. The SDD was highest for itraconazole followed by clotrimazole and least for fluconazole. Resistance was found highest to amphotericin B followed by nystatin, clotrimazole, itraconazole and no resistance was found for fluconazole [Table 5]. These findings are in accordance with the findings of Satana *et al.* 2010^[32] and in contrast to the reported findings by Sánchez-Vargas *et al.* 2005,^[6] Hamza *et al.* 2008.^[28]

The species *C. tropicalis*^[11] in HIV seropositive patients showed susceptibility to all the selected antifungal drugs and our findings were in accordance with Satana *et al.* 2010^[32] and Nweze *et al.* 2011.^[33]

Species *C. guilliermondii*^[11] [Table 6] isolated in HIV seropositive patients was susceptible to clotrimazole and

Table 4: Antifungal drug susceptibility against *C. albicans*

Drug	HIV seropositive patients (24) (%)			HIV seronegative individuals (25) (%)		
	S	SDD	R	S	SDD	R
Fluconazole	22 (91.6)	2 (8.4)	0 (0)	23 (92)	1 (4)	1 (4)
Amphotericin B	19 (79.1)	1 (4.1)	4 (16.6)	23 (92)	0 (0)	2 (8)
Nystatin	17 (70.8)	7 (29.1)	0 (0)	24 (96)	1 (4)	0 (0)
Clotrimazole	19 (79.1)	3 (12.5)	2 (8.4)	23 (92)	2 (8)	0 (0)
Itraconazole	15 (62.5)	8 (33.3)	1 (4.1)	23 (92)	1 (4)	1 (4)

C. albicans: *Candida albicans*, SDD: Susceptible dose dependent

Table 5: Antifungal drug susceptibility against *C. glabrata*

Drug	HIV seropositive patients (9) (%)			HIV seronegative individuals (1) (%)		
	S	SDD	R	S	SDD	R
Fluconazole	7 (77.7)	2 (22.2)	0 (0)	1 (100)	0 (0)	0 (0)
Amphotericin B	4 (44.4)	3 (33.3)	2 (22.2)	1 (100)	0 (0)	0 (0)
Nystatin	4 (44.4)	4 (44.4)	1 (11.1)	1 (100)	0 (0)	0 (0)
Clotrimazole	2 (22.2)	6 (66.6)	1 (11.1)	1 (100)	0 (0)	0 (0)
Itraconazole	1 (11.1)	7 (77.7)	1 (11.1)	1 (100)	0 (0)	0 (0)

C. glabrata: *Candida glabrata*, SDD: Susceptible dose dependent

Table 6: Antifungal drug susceptibility of *C. dubliniensis* and *C. guilliermondii* in HIV seropositive patients

Drug	<i>C. dubliniensis</i> (1) (%)			<i>C. guilliermondii</i> (1) (%)		
	S	SDD	R	S	SDD	R
Fluconazole	1 (100)	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)
Amphotericin B	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)	1 (100)
Nystatin	0 (0)	1 (100)	0 (0)	0 (0)	1 (100)	0 (0)
Clotrimazole	1 (100)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)
Itraconazole	1 (100)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)

C. dubliniensis: *Candida dubliniensis*, *C. guilliermondii*: *Candida guilliermondii*, SDD: Susceptible dose dependent

Table 7: Antifungal drug susceptibility against *C. tropicalis*

Drug	HIV seropositive patients (1) (%)			HIV seronegative individuals (8) (%)		
	S	SDD	R	S	SDD	R
Fluconazole	1 (100)	0 (0)	0 (0)	8 (100)	0 (0)	0 (0)
Amphotericin B	1 (100)	0 (0)	0 (0)	5 (62.5)	2 (25)	1 (12.5)
Nystatin	1 (100)	0 (0)	0 (0)	6 (75)	2 (25)	0 (0)
Clotrimazole	1 (100)	0 (0)	0 (0)	8 (100)	0 (0)	0 (0)
Itraconazole	1 (100)	0 (0)	0 (0)	6 (75)	2 (25)	0 (0)

C. tropicalis: *Candida tropicalis*, SDD: Susceptible dose dependent

itraconazole, SDD to fluconazole and nystatin whereas resistance to amphotericin B.

C. dubliniensis^[1] [Table 6] isolated in HIV seropositive patients was susceptible to fluconazole, itraconazole and clotrimazole, SDD to nystatin and resistance to amphotericin B. Findings of Hamza *et al.* 2008,^[28] Maninder *et al.* 2008, Badiie *et al.* 2010^[21] were favoring for susceptibility to fluconazole, itraconazole and clotrimazole, but their findings

are in contrast to our findings related to resistance for amphotericin B.

In the present study, the assessment of susceptibility, susceptibility dose dependent and resistance of the isolated *albicans* and non-*albicans Candida* species in HIV seronegative patients against the selected antifungal drugs were also assessed. As per the knowledge pertaining to the published scientific literature this study is the first of its kind in the literature carried out with above parameters in HIV seronegative patients and revealed the following facts.

Among the 39 species of *Candida* isolated in both HIV seropositive and HIV seronegative patients without the evidence of clinical oral candidiasis [Table 4]. *C. albicans* were highly predominant than non-*albicans* isolates. In HIV seronegative individuals, *C. albicans* showed the highest percentage of susceptibility against nystatin followed by fluconazole, amphotericin B, itraconazole and clotrimazole. Resistance was found highest to amphotericin B. Lone isolate of *C. glabrata* [Table 5] in HIV seronegative individuals showed susceptible to all selected antifungal drugs. *C. Tropicalis* showed highest percentage of susceptibility for fluconazole and clotrimazole followed by nystatin and itraconazole and least susceptibility to amphotericin B [Table 7]. It was observed that HIV seronegative patients showed higher susceptibility activity than HIV seropositive patients against itraconazole while as susceptibility dose-dependent and resistance was seen much higher in case of HIV seropositive patients.

CONCLUSION

Our results demonstrate that the tested antifungal agents showed good susceptibility activity for most isolates of both HIV seropositive and HIV seronegative groups; however, variability found among some isolates and resistance to antifungal agents emphasizes the need for antifungal drug susceptibility testing as a guide to the therapeutic prescription of antifungals agents.

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Conflicts of interest

There are no conflicts of interest.

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