Research Article

Identification, speciation of *Candida* using chrom agar and its antifungal susceptibility testing in various clinical samples

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Abstract

Introduction: Among species of *Candida*, both *C. albicans* and non albicans *Candida* species are often associated with serious fungal infections. Chromogenic media can identify candida species within 48 hours. Concern is rising about the emergence of antifungal resistance. **Aims and Objectives:** To isolate, identify and perform antifungal susceptibility of *Candida* from various clinical samples. **Material and Methods:** All samples were subjected to various mycological tests. Speciation of *Candida* species was done using both traditional methods and hicrome agar. Antifungal susceptibility testing was performed using disc diffusion method for fluconazole (25µg) and voriconazole (1 µg). **Results and Observations:** Out of 121 isolates, *Candida albicans* (51.24%) was the most common species. Among NAC, *C. tropicalis* (23.97 %) was most common. Out of 121 isolates, 51.24% were *Candida albicans* and 48.76% were non-*albicans Candida*. We obtained 100% sensitivity and specificity of HiCrome Candida differential agar for *C. albicans, C. tropicalis, C. krusei and C. dubliniensis* but sensitivity and specificity of Hicrome Candida differential agar for *C. glabrata* was 100% and 95.37% respectively. For fluconazole 81.82% *Candida* species were susceptible and 14.05% *Candida* species were resistant, whereas for voriconazole 90.91% *Candida* species were susceptible and 3.30% *Candida* species were resistant. **Conclusion:** Species identification using Hicrome Candida agar is rapid, technically simple, easy to interpret as compared conventional methods. Non-*albicans Candida* are more resistant to fluconazole than *C. albicans* particularly for *C. krusei* and *C. glabrata*.

Keywords: *Candida,* antifungal susceptibility.

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INTRODUCTION

Increased incidences of invasive fungal infections are likely associated with prolonged antibiotic therapy, invasive therapeutic procedures, radiotherapy, AIDS pandemic, organ transplant patients and cytotoxic chemotherapy.[1] *Candida* species are the most common cause of fungal infections worldwide.[2] *Candida* species are the fourth leading cause of health care associated infections and the third most common cause of central line-associated bloodstream infections. Among species of *Candida*, although *C. albicans* is most often associated with serious fungal infections, Other non albicans *Candida* species like *C. albicans, C.glabrata,C. parapsilosis and C.tropicalis*also have emerged as clinically important opportunistic pathogens.[3,4] Identification of yeast pathogens by traditional methods are labour intensive and requires several days and specific mycological media. Chromogenic media contain chromogenic substrates which react with enzymes secreted by target microorganisms to yield colonies of varying colours. This medium can be used as selective isolation medium for direct identification of clinical isolates of *Candida* species in less than 48 hours.[5,6] Concern is rising about the high incidence of infections caused by non-*albicansCandida* (NAC) species and the emergence of antifungal resistance. Among antifungal susceptibility tests, disc diffusion test has served as rapid,

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simple and cost-effective method for screening the susceptibility pattern of the yeasts.[7]

AIMS AND OBJECTIVES

1. To isolate *Candida* from various clinical samples.
2. To identify different species of *Candida.*
3. To find antifungal susceptibility pattern of *Candida.*

MATERIAL AND METHODS

After approval from institutional ethical committee, present study was conducted in tertiary care hospital. It was laboratory based prospective study from September 2012-October 2014. Various samples received in laboratory from patients of all age group and both sexes with suspected *Candida* infection were included in this study. Informed consent was taken from patients for sample collection. Clinical details were noted in the case record form. The specimens for laboratory investigation were collected under strict aseptic precautions. The various clinical specimens collected were oral swabs, ear swabs, vaginal swabs, urine, stool, CSF, sputum, blood, pus, nail scrapings etc. All the above samples were subjected to various mycological tests.

Direct Examination

a. **Wet mount for direct microscopic examination[8,9]:** Microscopic examination was done using 10% KOH. Attempt was made to identify the pseudohyphae and yeast cells.

b. **Gram stain[10]:** Gram stain was observed for gram positive yeast cells approximately 4-8 µm with budding and pseudohyphae.[8]

Culture

a. **Growth on Sabouraud dextrose agar[8]:** Sample was inoculated on Sabouraud dextrose agar with chloramphenicol and incubated at 25°C and 37°C then observed daily for growth after 24 hours to 72 hours.Colonies were identified by colony morphology and gram stain was done to confirm gram positive budding yeast cells.

Speciation of *Candida* species

a. **Germ tube test [10]:**A small portion of an isolate colony of the yeast to be tested was suspended in a test tube containing 0.5 ml human serum.The test tube was incubated at 35ºC for 2 hours.Under microscope,Filamentous extension from yeast cell with no constriction at the neck was considered as germ tube.

b. **Growth pattern on Cornmeal-Tween agar[8,9]:** Isolated colonies of Candida were inoculated on cornmeal-tween agar. The inoculated plates were incubated at 30ºC for 24-72 hours in a closed moisturized chamber. At the end of incubation period plates were examined microscopically (under 10x and 40x) at the edge of cover slip and the pattern of growth was observed to make a presumptive identification.

c. **Carbohydrate assimilation test[11,12] :**Yeast nitrogen base agar medium was melted in a boiling water bath and allowed to cool to up to 47-48ºC. Yeast suspension was made in 4 ml of distilled water with turbidity of suspension to match no. 5 McFarland standards. Yeast –agar mixture was poured in to sterile Petri dish and allowed to solidify at room temperature. Carbohydrate discs purchased from Hi Media, Mumbai were evenly spaced on the plate. Inoculated plates were incubated at 25°C and examined by indirect light every other day for 14 days. Any amount of growth around a disc was considered as yeast assimilated that carbohydrate. Species were identified based on pattern of carbohydrate assimilation.

d. **Growth on chromogenic agar [13]:** Isolated species were inoculated on HiCrome Candida differential agar and these agar plates were incubated at 37°C for 48 hours. The species were identified by characteristic colony colour as per HiMedia technical data M1297 A

- *C.albicans* – Light green coloured smooth colonies
- *C.tropicalis* - Blue to metallic blue coloured raised colonies
- *C.glabrata* - Cream to white smooth colonies
- *C.krusei* - Purple fuzzy colonies
- *C. dubliniensis* - Dark green[14]

Antifungal susceptibility testing[15]

Disc diffusion method was used for antifungal susceptibility testing. Mueller Hinton agar with 2% glucose and 0.5 µg/ml methylene blue was used. The antifungal agents fluconazole (25µg) and voriconazole (1 µg) are used for disc diffusion method. Zone diameter Interpretive Standards were followed as per CLSI M44-A2 guidelines.

Antifungal agent	Zone diameter (in mm)		
	Resistant (mm or less)	Susceptible-Dose dependent (mm)	Susceptible (mm or more)
Fluconazole (25µg)	14	15-18	19
Voriconazole (1 µg)	13	14-16	17

Controls used were: *C. albicans* ATCC 90028, *C. parapsilosis* ATCC 22019

RESUTS AND OBSERVATIONS

In present study, 121 isolates of *Candida species* were found. Species identification was done by both conventional method and Hicrome Candida differential agar. These strains were further tested for antifungal susceptibility to fluconazole and voriconazole by disc diffusion method. Most of the *Candida* isolates were common in the age group of 21-40 years (39.67 %).Majority of *Candida* isolates were seen in female patient (53.72%) compared to male patients (46.28%). Total number of *Candida* species isolated was 121. Out of 121 isolates, *Candida albicans* (51.24%) was the most common species. Among NAC, *C.tropicalis* (23.97 %) was most common followed by *C. glabrata* (10.74%), *C.krusei* (7.44 %), *C. parapsilosis* (4.13 %) *and C.dubliniensis* (2.48 %).Out of 121 isolates, 51.24% were *Candida albicans* and 48.76% were non-*albicans Candida*. In our study conventional method was considered as reference method for speciation. All species were correctly identified by Hicrome Candida differential agar as compared to conventional methods within 48 hours, except 5 species of *C.parapsilosis* (identified by conventional method) which were identified by Hicrome Candida agar as *C. glabrata*.Five species of *C. parapsilosis* were identified as *C. glabrata* by Hicrome Candida agar which were considered as false positive. We obtained 100% sensitivity and specificity of HiCrome Candida differential agar for *C. albicans, C. tropicalis, C. krusei and C. dubliniensis* but sensitivity and specificity of Hicrome Candida differential agar for *C. glabrata* was 100% and 95.37% respectively. For fluconazole 81.82% *Candida* species were susceptible and 14.05% *Candida* species were resistant, whereas for voriconazole 90.91% *Candida* species were susceptible and 3.30% *Candida* species were resistant. Thus voriconazole was found to be effective as compared to fluconazole. For fluconazole, out of 62 *C.albicans* species 57 were susceptible, 3 were susceptible dose dependent and 2 were resistant, where as among 59 non-*albicansCandida* species, 42 were susceptible, 2 were susceptible dose dependent and 15 were resistant. For voriconazole, out of 62 *C.albicans* species, 60 were susceptible and among remaining 2 *C.albicans,* one was susceptible dose dependent and other was resistant, where as among 59 non-*albicans Candida* species, 50 were susceptible, 3 were resistant and remaining 6 were susceptible dose dependent. *C. albicans* (91.94%) were found to be more susceptible to fluconazole as compared to non-*albicans Candida* (71.19%). This was statistically highly significant ($p<0.001$) where as both *C. albicans* (96.77%) and non-*albicans Candida* (84.57%) were highly susceptible to voriconazole as statistically ($p>0.05$) there was no difference between their susceptibility.

Table 1: Identification of various species of *Candida* by conventional method and Hicrome Candida differential agar

Candida species	Conventional method	Hicrome agar
C.albicans	62	62
C.tropicalis	29	29
C.glabrata	13	18
C.krusei	9	9
C.parapsilosis	5	-
C.dubliniensis	3	3

Table 2: Performance of Hicrome Candida differential agar as identification medium compared with conventional method

Candida species (121)	True positive	True negative	False positive	False negative	Sensitivity (%)	Specificity (%)
C.albicans	62	59	0	0	100	100
C.tropicalis	29	92	0	0	100	100
C.glabrata	13	103	**5**	0	100	**95.37**
C.krusei	9	112	0	0	100	100
C.dubliniensis	3	118	0	0	100	100

Table 3: Susceptibility patterns of *Candida* species to fluconazole (25µg) and voriconazole (1 µg)

	Fluconazole (25 µg)			Voriconazole (1 µg)		
	S n(%)	S-DD n (%)	R n (%)	S n (%)	S-DD n (%)	R n (%)
C.albicans (62)	57(91.94)	3(4.84)	2(3.22)	60(96.77)	1(1.61)	1(1.61)
C.tropicalis (29)	25(86.21)	1(3.45)	3(10.34)	26(89.65)	2(6.90)	1(3.45)
C.glabrata (13)	10(76.92)	1(7.69)	2(15.39)	11(84.62)	1(7.69)	1(7.69)
C.krusei (9)	0	0	9(100)	6(66.67)	2(22.22)	1(11.11)
C.parapsilosis (5)	4(80)	0	1(20)	4(80)	1(20)	0
C.dubliniensis (3)	3(100)	0	0	3(100)	0	0
Total	**99(81.82)**	**5(4.13)**	**17(14.05)**	**110(90.91)**	**7(5.79)**	**4(3.30)**

S- Susceptible, S-DD- Susceptible dose dependent, R-Resistant

Table 4: Susceptibility patterns of *C.albicans and non-albicans Candida* species to fluconazole (25µg) and voriconazole (1 µg)

Drug	Fluconazole (25 µg)		Voriconazole (1 µg)	
	C.albicans n (%)	*Non albicans Candida* n (%)	*C.albicans* n (%)	*Non albicans Candida* n (%)
Susceptible	57 (91.94)	42(71.19)	60(96.77)	50(84.75)
Resistant	2(3.22)	15(25.42)	1(1.61)	3(5.08)

Fisher's exact test $p < 0.001$ (for fluconazole), Fisher's exact test $p > 0.05$ (for voriconazole)

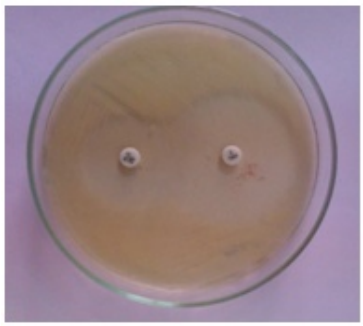

Figure 1: Antifungal susceptibility testing Corn meal- Tween agar

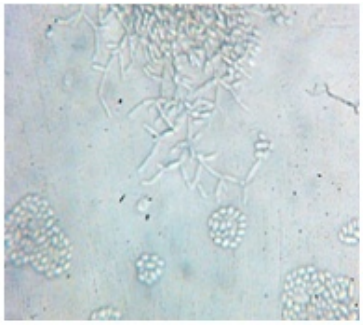

Figure 2: Growth pattern on

(Extensive branched pseudomycelium with chains of elongate cells giving cross match stick appearance) (40x)

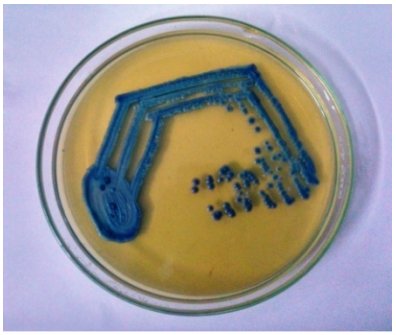

Figure 3: C. tropicalis showing blue colonies on Hicrome Candida

DISCUSSION

Present study was based on isolation, speciation and antifungal susceptibility testing of *Candida* in various clinical samples. Total 121 *Candida* species were isolated from various clinical samples. In present study, most of the *Candida* isolates (39.67%) were predominant in the age group of 21-40 years which correlates with study conducted by Dharwad S *et al*[16] and Jaggi T *et al*.[17] We found female preponderance in our study which was concordant with the study conducted by Sajjan AC *et al*[18] and Dharwad S *et al*.[16] Among the various clinical isolates of *Candida* species we obtained *C.albicans* (51.24%) as the most common isolate followed by *C. tropicalis* (23.97%), *C. glabrata* (10.74%), *C. krusei* (7.44%), *C. parapsilosis* (4.13%) and *C.dubliniensis* (2.48%). While non-*albicans Candida* were 48.76%. In respect to predominance of *C. albicans* isolates and distribution of species, similar results were found in study conducted by Pfaller MA *et al*,[19] Sajjan AC *et al*[18]and Mondal S *et al*.[20] Factors like increased use of antifungal drugs, use of broad spectrum antibiotics, long term use of catheters and increase in the number of immunocompromised patients contributes to the emergence of non-*albicans Candida* species.[21] For differentiation among different species of *Candida* conventionally germ tube test, growth pattern on cornmeal agar and sugar assimilation tests are being used which are technically difficult, time consuming and difficult to interpret which may take 72 hours to two weeks for species identification.[5,22] Chromogenic agar is technically simple, easy to interpret and rapid method to differentiate among different *Candida* species. It facilitates the detection and identification of *Candida* species and provides result in 24-48 hours.Among the newer tests, chromogenic agar is rapid and cost effective as compared to other expensive systems like API systems, Vitek 2 ID system and molecular methods.[23] We obtained 100% sensitivity and specificity of HiCrome Candida differential agar for *C.albicans, C.tropicalis, C.krusei and C.dubliniensis* but sensitivity and specificity of Hicrome

Candida differential agar for *C.glabrata* was 100% and 95.37% respectively. However our study correlates with following studies showing high sensitivity and specificity of chromogenic agar.

Species	*C.albicans*		*C.tropicalis*		*C.glabrata*		*C.krusei*	
	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity
Yucesoy M *et al*[24] (2003)	99.4%	100%	97%	100%	98.9%	100%	100%	100%
Daef E*et al*[25]	100%	98.9%	100%	100%	100%	99%	100%	100%
Yucesoy M *et al*[26] (2005)	100%	100%	100%	100%	90%	100%	100%	99.5%
Our study	**100%**	**100%**	**100%**	**100%**	**100%**	**95.37%**	**100%**	**100%**

For *C. glabrata*, specificity of Hicrome Candida agar was 95.37%, as 5 species of *C.parapsilosis* (identified by conventional method) were identified by Hicrome Candida agar as *C. glabrata*. Shettar SK *et al*[27] reported that on Hicrome Candida agar *C.parapsilosis* gave same cream colour as that of *C.glabrata*. Ghelardi E *et al* [28] studied Chromogenic Candida agar (CCA, Oxoid, basingtoke, UK) for identification of *Candida* species. According to their study, this medium didn't allow discrimination of *C. glabrata* and *C.parapsilosis*. This may be because *of C. glabrata, C. kefyr, C. parapsilosis*and *C.lusitaniae*appear as a variety of beige/brown/yellow colours due to the mixture of natural pigmentation and some alkaline phosphatase activity.[29] *C.glabrata* and *C. parapsilosis* can be easily differentiated from growth pattern on Cornmeal agar as *C. glabrata* doesn't produce pseudohyphae. Thus, the combination of Cornmeal agar and Hicrome Candida agar can be used for early identification of *C. glabrata*.[27] In our study *C. krusei* was 0% susceptible to fluconazole while other species were susceptible as follows: *C. dubliniensis* 100%, *C. albicans* 91.94%, *C.tropicalis* 86.21%, *C. glabrata* 76.92% and *C. parapsilosis* 80% (table 6).This is in correlation with study done by Pfaller MA *et al* [19] Oberoi JK *et al* [30]Lee JS *et al* [31]. We observed that there was increased fluconazole resistance among non-*albicans Candida* (25.42%)compared to *C.albicans* (3.22%) (Table 4). 100% resistance of *C.krusei* to fluconazole can be explained byintrinsic resistance in *C.krusei* as a result of impaired binding of fluconazole to 14 α-demethylase.[32]Higher fluconazole resistance in *C.glabrata* may be result of the expression of multidrug efflux pump and also as haploid nature of *C. glabrata* genome makes these pathogen particularly well suited for acquiring and expressing MDR resistance traits in the presence of drug pressure.[32,33] In our study *C. dubliniensis* (100%), *C. albicans* (96.77%), *C.tropicalis* (89.65%), *C. glabrata* (84.62%), *C. parapsilosis* (80%) and *C. krusei* (66.67%) were susceptible to voriconazole. Our study correlates with studies done by Oberoi JK *et al*[30] Pfaller MA *et al* [19] Comparing resistance pattern of fluconazole and voriconazole (table 4) among all *Candida* isolates 14.05% were resistant to fluconazole while 3.30% were resistant to voriconazole. Sajjan AC *et al*[18] and Mondal S *et al*[20] reported 12.6% and 18% resistance to fluconazole respectively. According to Pahwa *et al*[34] study, 1% *C. Albicans* and 3.6% non *albicans Candida* were resistant to voriconazole. Voriconazole seemed to be superior to fluconazole with a better susceptibility pattern. This may be due to the more effective binding of voriconazole to cytochrome P-450 isoenzyme of *Candida* species.[35]

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