ORIGINAL ARTICLE

Speciation, Biofilm Formation and Antifungal Susceptibility of *Candida* Isolates from Clinically Diagnosed Patient of UTI in a Tertiary Care Hospital

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Abstract

Introduction: The incidence of the urinary tract infections caused by *Candida* species, are becoming more common. Recently, an increase in the incidence of infection caused by fungi especially non albicans candida species (NAC) has been reported. Several virulence factors like biofilm formation, toxin production and presence of adhesins contribute to its pathogenesis.

Objectives: This study was undertaken to determine species distribution, biofilm formation and in-vitro antifungal susceptibility of candida isolated in our tertiary care hospital.

Method:Eighty seven clinical isolates obtained from urine specimens were subjected to wet mount, Gram's stain and cultured on Sabouraud's Dextrose agar (SDA) medium. Conventional method for yeast identification was done. Biofilm forming ability of each isolate was detected using microtitre plate method. Antifungal susceptibility against posaconazole, amphotericin-B, fluconazole, itraconazole, ketoconazole, 5-flucytosine, voriconazole, and caspofungin was tested using Sensititre® Yeastone® (Trek diagnostic systems).

Results & Discussion: Out of 87 candida isolates, 31.03% (n=27) were *C. albicans* and 68.97% (n=60) were non albicans candida species (NAC). Among 60 NAC, *C. kruseii* 29.89% (n=26), *C. glabrata* 24.14% (n=21), *C. tropicalis* 14.94% (n=13). Among all isolates, 36.78% (n=32) were biofilm producers and biofilm positivity more among *C. albicans* 55.56% (n=15) as compared to NAC 28.33% (n=17) (*P*-value<0.002). The maximum positivity was observed with isolates from plastic devices (61.8%). The minimum inhibitory concentrations of all antifungal drugs against all isolates were within susceptible range except for fluconazole which was resistant to *C. kruseii*.

Conclusion:*C. albicans* remains the major isolate from urine samples and also biofilm formation as a virulence factor might have a higher significance for *C. albicans* than for NAC and its ability to form biofilm is intricately linked with ability of organisms to adhere, colonize and subsequently cause infection.

Introduction

Candida species are ubiquitous yeast found on many plants and are members of the normal flora of alimentary tract of mammals and mucocutaneous membranes of humans. They exist predominantly in the unicellular form with both sexual and asexual forms and show thin walled ovoid cells (blastospores) that reproduce by budding. Out of more than 150 species of candida, only nine species are regarded as frequent pathogens for humans.[1]

Candida is the sixth most common isolated nosocomial pathogen, especially from urinary tract.[2]

The finding of candiduria in a patient with or without symptoms should be requires a careful evaluation, which should proceed in a logical fashion.[3] *Candida* UTI are increasingly common due to prolonged antibiotic use, indwelling urinary catheters and immunocompromised individuals.[4]

Moreover drug resistance is a major cause of treatment failure in these patients. Among the different antifungal agents, resistance to the polyene compounds has remained an uncommon problem. But resistance to flucytosine and azoles now appears to be increasingly important in some group of patients, especially after the widespread use of fluconazole for extended periods.[5]

Several virulence factors like biofilm formation and presence of adhesins contribute to its pathogenesis. Biofilms are universal, complex, interdependent communities of surface-associated microorganisms, enclosed in an exopolysaccharide matrix occurring on any surface, including medical devices. Since biofilms are notoriously difficult to eliminate and serve as a source of recalcitrant infections, their study is highly relevant for public health.[6] Biofilm drug resistance is a phenomenon of great clinical relevance explaining persistence of infection even in the face of an appropriate antifungal therapy.[7, 8]

In view of the above observations, this study was carried out with an aim to study species distribution of *Candida* isolates among UTI patients, biofilm formation as a virulence factor and their antifungal susceptibility pattern.

Material and Methods

The prospective cross sectional study was conducted, after obtaining approval from institutional ethical committee and fully informed and voluntary consent were obtained from the patient and / or their attendants,

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in the Mycology section of Department of Microbiology, G.R. Medical College, Gwalior, Madhya Pradesh during the period of one year from Jan, 2015 to Dec, 2015.

All urine specimens of suspected case of UTI patients attending OPD, or admitted in various wards and ICU, among them which are culture positive for fungal growth (Yeast only) were included in the study. And those specimens which are culture negative (No growth) or culture positive for bacterial growth were excluded.

Clean catch mid-stream urine samples were collected in a sterile wide mouth leak-proof container. In catheterized patients' catheter clamp technique were used for sample collection. Samples were transported to the laboratory as soon as possible with mean transport time of one hour, if delay of 2 to 4 hours samples were refrigerated and if more than 4 hour then samples were discarded and fresh sample were collected. Samples were processed as shown in Flow Chart 1 with their proper requisition form including age, sex, past medical and surgical history, presence of an indwelling urinary catheter, patient on antibiotics and duration of stay in ICU etc.

A total of 87 Candida isolates were recovered from 3381 urine specimens as a part of routine diagnostic procedures (Figure 1). These patients had no history of antifungal drug exposure prior to the collection.

Fig. 1: Colony of Candida spp. on blood agar

Fig. 2: Colony of *Candida* spp. on SDA

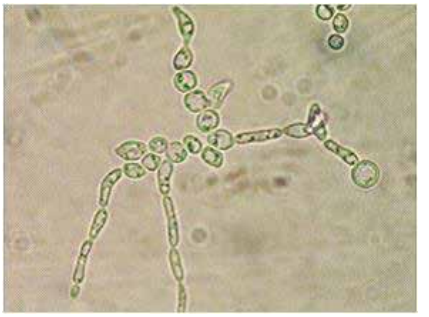
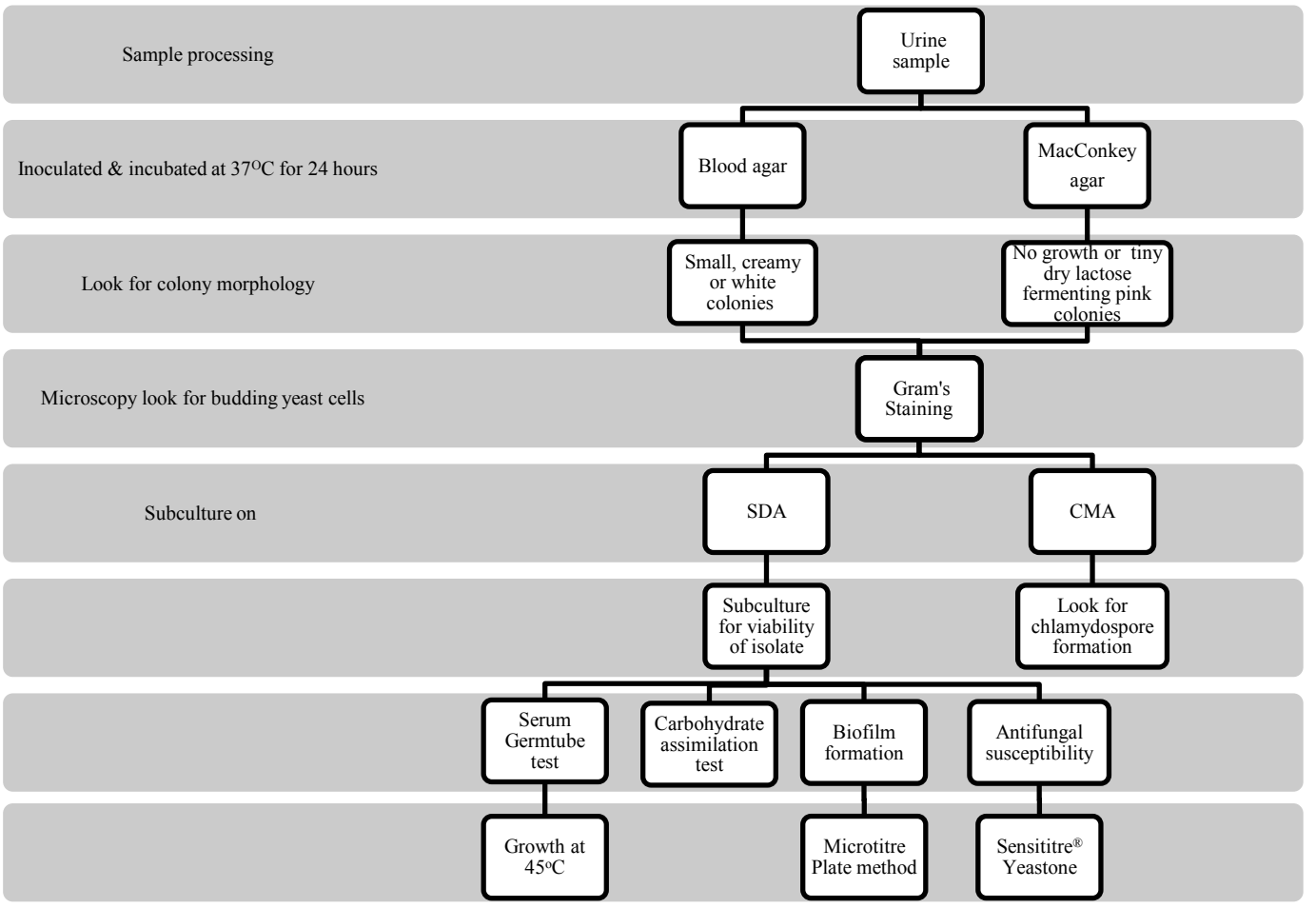


Fig. 3: Chlamydospore on corn meal agar observed under 40X



Flow chart 1: Isolation and identification

These candida isolates were subcultured on Sabouraud's Dextrose Agar (HiMedia Laboratories Pvt. Ltd. Mumbai, India) for study (Figure 2) Identification was carried out by performing Gram's stain, Germ tube test,[9] Chlamydospore production test[10] (Figure 3), Growth at 45°C[9] and Carbohydrate assimilation test as per the CLSI guidelines. Antifungal susceptibility against posaconazole, amphotericin-B, fluconazole, itraconazole, ketoconazole, 5-flucytosine, voriconazole, and caspofungin was tested using Sensititre® Yeastone® (Trek diagnostic systems) as per manufacturer instructions.

Biofilm production (Microtiter plate method)[6,11,12] 24 hour old isolates on Sabouraud dextrose agar (SDA) were washed and suspended in sterile saline equivalent to 0.5 McFarland standards. 20 µl of isolate suspension and 180 µl of Sabouraud dextrose broth (SDB) with 8% glucose were inoculated in each well of flat bottom polystyrene micro-titer plate (Figure 4) and incubated at 37°C for 24 hours without agitation. Then wells were washed twice with 0.15 M phosphate buffer saline (PBS) by Thermo Scientific Well wash machine. And 200µL of PBS was added to each well and spectrophotometric readings were performed twice at 405 nm with SkanIt Software (version 4.1) for Microplate Reader (Thermo Fisher Scientific). The percent transmittance (%*T*) was calculated by subtracting the %*T* value for each test sample from the %*T* value for the reagent blank to obtain a measure of the amount of light blocked passing through the wells ($\%T_{block}$). Biofilm production by each isolate was scored as given given below in the Table 1.

- Mean percent transmittance (%T) of test = (%T1 + %T2)/2
- Mean %T of test – mean %T value of Blank well = $\%T_{block}$ of that test.

Fig. 4: Flat bottom polystyrene Microtitre plate for biofilm

Table 1: Biofilm production score chart

Interpretation	$\%T_{block}$
Negative	< 5
1+	5-20
2+	21-35
3+	36-50
4+	≥ 50

Table 2: Percentage of biofilm producer candida

	C. albicans	NAC	Total
Total	27	60	87
Biofilm Production	15 (55.56%)	17 (28.33%)	32

[This difference was statistically significant (p-value <0.002)]

Result and Discussion

A total of 87 patients with candida UTI were evaluated during the study period. Organism causing infection included *Candida albicans* (31.03%; 27 patients), *C. kruseii* (29.89%; 26 patients), *C. glabrata* (24.14%; 21 patients) and *C. tropicalis* (14.94%; 13 patients). Graph 1

Thirty two (36.78%) of 87 patients were infected by biofilm forming isolates, as assessed by the %T method. Biofilm production by *C. albicans* was significantly more frequent (55.56%) than that by all other NAC (28.33%; *P*-value < 0.002). Among the species, most commonly isolated from candida UTI patients (*C. albicans, C. kruseii, C. tropicalis* and *C. glabrata*), biofilm production was most frequently observed for isolates of *C. tropicalis* (69.23% [9 of 13]), followed by *C. albicans* (55.56% [15 of 27]), *C. glabrata* (19.04% [4 of 21]) and *C. kruseii* (15.38% [4 of 26]) Graph 2 and Table 2.

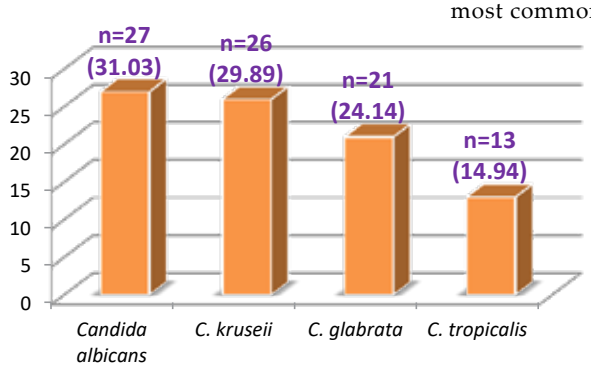
Isolates of *Candida* species were all found susceptible in vitro to amphotericin B, caspofungin, posaconazole, fluconazole, itraconazole, ketoconazole, 5 flucytosine, voriconazole and, except for all *C. tropicalis* and all *C. krusei*, to fluconazole antifungal agents as shown in Table 3.

Patient demographic and clinical characteristics-

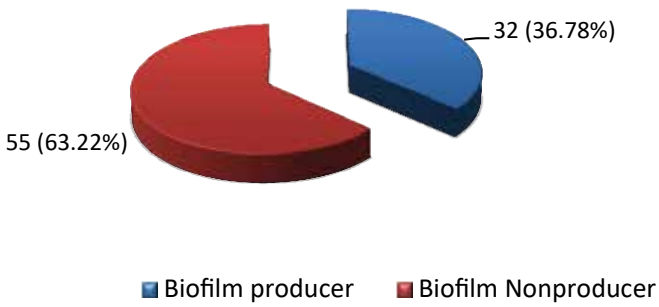
Of 87 patients with candida UTI having age of 42.9 ± 13.2 (mean age ± standard deviation), male to female ratio of 1:2 as shown in Tables 4 and 5.

Discussion

Candida species are now the sixth most common cause of nosocomial urinary tract infection worldwide.[1] Variables, such as patient age, underlying disease, location in hospital (ICU or non- ICU), indwelling urinary catheter and higher antibiotic exposure may influence the frequency and rank order of *Candida* species causing urinary tract infections.[13,14] in this study, the distribution of *Candida* species was similar to reports from other countries,[15,16] with *C. albicans* being the fungal species most frequently isolated from urine but there were significant increases found in the prevalence of non-*C. albicans Candida* species causing urinary tract infection. In this study, among the non- *C. albicans Candida, C.krusei* (n=26, 29.89%) was predominating isolated species followed by *C. Glabrata* (n=21, 24.14%), and *C. tropicalis* (n=13, 14.94%). In the



Graph 1: Bar diagram of distribution of *Candida* species among UTI cases



Graph 2: Biofilm production by *Candida*

Table 3: Antifungal susceptibility pattern of *Candida* spp

Candida species	PZ	AMB	FZ	IZ	KZ	5FC	VOR	CAS
C.albicans	S (1)	S (0.5)	S (≤1)	S (≤0.25)	S (0.5)	S (≤1)	S (≤0.12)	S (≤0.25)
C. kruseii	S (1)	S (0.5)	R (8)	S (≤0.25)	S (0.5)	S (≤1)	S (≤0.12)	S (≤0.25)
C.glabrata	S (1)	S (0.5)	S (≤1)	S (≤0.25)	S (0.5)	S (≤1)	S (≤0.12)	S (≤0.25)
C. tropicalis	S (1)	S (0.5)	R (8)	S (≤0.25)	S (0.5)	S (≤1)	S (≤0.12)	S (≤0.25)

(PZ=Posaconazole, AMB=Amphotericin B, FZ=Fluconazole, IZ=Itraconazole, KZ=ketoconazole, 5FC=5 Flucytosine, VOR=Voriconazole, CAS=Caspofungin)

present study, biofilm production was found to occur most frequently among C. albicans (55.56%) than in among NAC (28.33%). This finding is in concordance to an earlier report that suggested that pathogenic C. albicans were more likely to produce biofilms than among NAC[17] The explanation for this observation is not clear and warrants further study, but it is possible that some medical procedures might consistently impact the risk of developing urinary tract infection, caused by a fungal isolate capable of forming biofilm over the years. In this study, we used polystyrene plates to grow biofilms. Although a valid assay for biofilm formation in *C. albicans* based on polystyrene microtiter plates has now been established and standardized,[12] it is possible that use of other materials, such those used for indwelling devices, including catheters (e.g., silicone elastomer), may give results different from those we obtained here, perhaps because the polystyrene may not reflect exactly the ability to form a biofilm in vivo. In addition to the substrate material, it is important to note that other environmental parameters, such as substrate preconditioning solutions (i.e., those mimicking physiologic conditions, such as the presence of serum or saliva) and growth media can affect biofilm production.[18] Antifungal resistance was rarely found in our study and was restricted to fluconazole resistance for all isolates of *C. krusei* and *C. tropicalis* only. In the present study it was observed that incidence of Candiduria was reported higher among the females (66.67%) than males (33.33%). Mahajan A. *et al*[15] reported 74% females and 26% males had Candiduria. In a study, N. Safdar *et al*[16]found that 77% females had candiduria. N. Jain *et al*[19] observed that 77.4% females had candiduria. However Kobayashi *et al*[14] reported female incidence to be 57.8%. Kauffman CA *et al*[20] reported 59.9% females with candiduria. Hence, all studies done in different parts of the world, show that females have more predilections towards candiduria, most probably due to short urethra in females.

In the present study common predisposing condition included urinary catheter 61.8%, patients using antibiotics 54.4%, diabetes in 44.5%, ICU stay in 26.4%, age between 31 to 60 year and sex that was affected more is female that is 66.67%. According to Navin Paul *et al*[13]incidence of various predisposing factors was catheterization 66.6%, intake of antibiotics 47.61%, diabetes 38.09% and surgery in 38.09%. That is in accordance to the present study. Kobayashi *et al*[14] reported incidence of various predisposing factors was: intake of antibiotics 100%, urinary catheter was present in 84.4%, surgical procedure in 66.7%.

Conclusion

C. albicans remains the major isolate from urine samples and also biofilm formation as a virulence factor might have a higher significance for *C. albicans* than for NAC and its ability to form biofilm is intricately linked with ability of organisms to adhere, colonize and subsequently cause infection.

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Table 4: Age and Sex wise distribution of fungal isolates (n=87)

Age group (in years)	Male n (%)	Female n (%)
0-10	1 (3.45)	1 (1.72)
11-19	1 (3.45)	3 (5.17)
20-35	6 (20.69)	10 (17.24)
36-50	13 (44.83)	23 (39.66)
51-70	8 (27.58)	21 (36.21)
>71	0 (0)	0 (0)
Total	29 (33.33)	58 (66.67)

Table 5: Epidemiological characteristics of 87 patients with Candida UTI

Variable	*C. albicans* (n=27)	NAC (n=60)	P*-value
Age in years (mean ± SD)	42.9 ± 13.2		
Male sex	11 (40.74)	18 (30)	P=0.32
Female sex	16 (59.26)	42 (70)	P=0.32
In the ICU at diagnosis	11 (40.74)	12 (20)	P=0.04
Diabetes	15 (55.56)	24 (40)	P=0.17
On antibiotics	20 (74.07)	27 (45)	P=0.01
Indwelling urinary catheter	17 (62.96)	37 (61.66)	P=0.91
Biofilm formation	15 (55.56)	17 (28.33)	P=0.01

**P* value comparing the value for patients infected with *C. albicans* to the value for patients infected with non albicans *Candida* species.

References

1. Lal BY, Kalyani M. Phenotypic Characterization of Candida species and their antifungal susceptibility from a tertiary care centre. *JPBMS* 2011; 11:1-5.
2. Andy IM Hoepelman - Infectious Disease-Jonathan Cohen, William G Powderly –2nd edition (Harcourt publishers' limited-2004)-Chapter-237.
3. Kauffman CA, Fisher JF, Sobel JD, *et al*. Candida urinary tract infections- diagnosis. *Clin Infect Dis* 2011; 52:452-6.
4. Lundstrom T, Sobel J. Nosocomial Candiduria: A Review. *Clinical Infectious Diseases* 2001; 32:1602–7.
5. Mahajan A, Kaur N, Kaur A, *et al*. Isolation, identification and antifungal susceptibility pattern of *Candida* Spp isolated from UTI cases in a tertiary care hospital. *Sch J App Med Sci* 2015; 3:2146-52.
6. Kumar CPG, Menon T. Biofilm production by clinical isolates of Candida species. *Medical Mycology* 2006; 44:99-101.
7. Kojic EM, Darouiche RO. Candida infections of medical devices. *Clin Microbiol Rev* 2004; 17:255-267.
8. Douglas LJ. Medical importance of biofilms in Candida infections. *Rev Iberoam Micol* 2002; 19:139-143.
9. Marinho SA, *et al*. Identification of *Candida* spp. by phenotypic tests and PCR. *Brazilian Journal of Microbiology* 2010; 41:286-294.
10. Balish E. Chlamydospore production and germ-tube formation by auxotrophs of *Candida albicans*. *Applied Microbiology* 1973; 25:615-620.
11. Tumbarello M, Posteraro B, *et al*. Biofilm production by *Candida* species and inadequate antifungal therapy as predictors of mortality for patients with candidemia. *Journal of Clinical Microbiology* 2007; 45:1843–50.
12. Ramage GK, Walle V, *et al*. Standardized method for in vitro antifungal susceptibility testing of *Candida albicans* biofilms. *Antimicrob. Agents Chemother* 2001; 45:2475–79.
13. Paul N, Mathai E, Abraham OC, *et al*. Emerging microbiological trends in candiduria. *Clin Infect Dis* 2004; 39:1743-4.
14. Kobayashi CC, de Fernandes OF, Miranda KC, *et al*. Candiduria in hospital patients: a study prospective. *Mycopathol* 2004; 158:49-52.
15. Mahajan A, Kaur N, Kaur A, *et al*. Isolation, identification and antifungal susceptibility pattern of *Candida* spp isolated from UTI cases in a tertiary care hospital. *Sch J App MedSci* 2015; 3:2146-52.
16. Safdar N, Slattery WR, Knasinski V, *et al*. Predictors and outcomes of candiduria in renal transplant recipients. *Clin Infect Dis* 2005; 40:1413-21.
17. Hawser SP, Douglas LJ. Biofilm formation of *Candida* species on the surface of catheter materials in vitro. *Infect Immun* 1994; 62:915-921.
18. Kuhn DM, Chandra J, Mukherjee PK, et al. Comparison of biofilms formed by *Candida albicans* and *Candida parapsilosis* on bioprosthetic surfaces. *Infect. Immun* 2002; 70:878–888.
19. Jain N, Kohli R, Cook E, *et al*. Biofilm formation by and antifungal susceptibility of candida isolates from urine. *Applied and Environ Microbiol* 2007; 73:1697-703.
20. Kauffman CA, Vazquez JA, Sobel JD, *et al*. Prospective multicenter surveillance study of funguria in hospitalized patients. *Clin Infect Dis* 2000; 30:14- 8.