

Prevalence of Candidial Infections with their Antifungal Susceptibility Pattern in a Tertiary Care Hospital

Neelusree Prabhakaran, S.S.M. Umamageswaria*,
Muthumari and Kalyani Mohan

Department of Microbiology Saveetha Medical College and Hospital,
Saveetha Nagar, Thandalam, Kancheepuram, Chennai - 602105, India.

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Morbidity and mortality due to the rise in the incidence of fungal infections has increased significantly. The genus *Candida* is the most common cause of fungal infection worldwide. Recent evidence suggests that majority of infections produced by this pathogen are associated with biofilm growth. Biofilms are colonies of microbial cells encased in a self produced organic polymeric matrix. The biofilm production is more important for *Non Albicans Candida* (NAC); as *C. albicans* possess many other mechanisms to establish infections. Correct identification of *Candida* species has gained importance due to persistent rise in infections caused by NAC. The development of resistance in *Candida* is also raising due to the increase in immunocompromised status and critical patients. To study the prevalence of *Candida* species, biofilm formation for *Candida* isolates and their antifungal susceptibility pattern in a tertiary care centre. Sixty eight clinical isolates of *Candida* were collected from various clinical samples in a tertiary care centre, Chennai for a period of six months. Identification of *Candida* species was done by germ tube tests, test for chlamydoconidia production, sugar assimilation tests and CHROM agar isolation method. Antifungal susceptibility tests against fluconazole, Amphotericin B and Flucytosine were performed by disc diffusion method and for the resistant *Candida* strains Minimum inhibitory concentration (MIC) was performed using E-strip for fluconazole, Amphotericin B and Flucytosine. Out of 68 isolates *C. albicans* tops the first 67.6%, *C. tropicalis* ranks the next most predominant species 17.64% and the least common isolates *C. parapsilosis* 8.8% and *C. glabrata* 5.8%. Candidial infections were more commonly observed in female patients 57.3% and hospitalized in- patients 69.1% with increased number from non – catheterized urine samples. The Fluconazole susceptibility using disc diffusion showed 69% of *C. albicans* and 58.3% of *C. tropicalis* and 75.5% of *C. parapsilosis* were sensitive to fluconazole and sensitivity to Flucytosine were as follows *C. albicans* (86.60%) *C. tropicalis* (75%) *C. parapsilosis* and *C. glabrata* (100%). Though a higher degree of antifungal resistance noted among the various *Candida* species, still can be used as a treatment drug for critical care patients as the MIC of those anti fungal drugs were found to be in the susceptible range. Isolation of NAC from clinical samples, their biofilm formation and antifungal resistance are on a rise, they should be treated as a pathogen and not dismissed.

Keywords: CHROM agar, Minimum Inhibitory Concentration, Biofilms, *Candida* species.

Candida spp are among the members of the normal flora of skin, mucous membrane and gastrointestinal tract. In the recent years due to

advances in medical technologies there is an increase in number of immunocompromised individuals which has resulted in rise in the incidence of fungal infections especially those due to *Candida* species¹. There are more than 200 spp, of which *C. albicans* is the most common, other

* To whom all correspondence should be addressed.

commonly isolated species are *C. glabrata*, *C. parapsilosis*, *C. tropicalis* and *C. krusei*. Overgrowth of these fungus results in Candidiasis (Candidosis) which can cause superficial, subcutaneous and deep mycosis. The incidence of Candidemia in intensive care unit (ICU) patients is also on a rise. In addition, hospital acquired infections by *C. albicans* have become a major health concern². The generous use of broad spectrum antibiotics and novel molecules to some extent have checked the infectious disease, but simultaneously have paved way for new advanced and modalities of opportunistic pathogen viz., *Candida*. Diabetes mellitus, the leading endocrine dysfunction and HIV infection, the new world pandemic provided a potent soil for Candidial infection.²

Infection caused by NAC may be indistinguishable but the importance in their detection lies in the fact that many *non albicans* species are inherently resistant or likely to acquire resistance to the routinely used anti-fungal drugs. One of the reasons would be biofilm which may form on the surface of implantable medical devices. Biofilms are colonies of microbial cells encased in a self-produced organic polymeric matrix and represent a common mode of microbial growth. Recently, microbial biofilms have gained prominence because of the increase in infections related to indwelling medical devices.⁽³⁾ The advantages of forming a biofilm for the organism include protection from the environment, nutrient availability, metabolic cooperation and acquisition of new genetic traits.⁴ Biofilms may help maintain the role of fungi as commensal and pathogen, by evading host immune mechanisms, resisting antifungal treatment and withstanding the competitive pressure from other organisms. Consequently, biofilm related infections are difficult to treat.⁵ The biofilm production is also associated with high level of antimicrobial resistance of the associated organisms.⁶ The proportion of infections due to NAC is persistently increasing the need for a correct identification of *Candida* isolates at the species level. Although *C. albicans* remains the most common fungal isolate recovered from blood, recent reports indicate a trend toward an increasing prevalence of infections caused by NAC.⁷⁻¹⁰ The proportion of biofilm production is

much higher among isolates of NAC species recovered from blood than other sites.¹¹

MATERIALS AND METHODS

A descriptive study was undertaken in a tertiary care centre, Chennai for a period of six months. *Candida* strains were collected from various clinical samples like urine, exudates, blood and sputum (confirmed as *Candida* by observing the characteristic budding yeast cells with pseudohyphae in gram stain and 10% KOH mounts). Only pure growth of *Candida* spp (no other aerobic pathogens) excluding normal flora were included in this study. The confirmed *Candida* isolates were further speciated by germ tube test, chlamydoconidia production test on corn meal agar, sugar assimilation tests for Dextrose, Lactose, Sucrose, Trehalose and Maltose and by the colour of growth on HiChrom *Candida* agar isolation method, Hi media laboratories Pvt. Ltd, India.

Biofilm formation: Presterilized polystyrene, flat bottom 96 well microtitre plate were used.

Procedure:

Step 1

Adhesion phase

100 ml of standardized cell suspension (10⁷ cells/ml) was transferred into each well of plate with a sterile pipette and Incubated for 1.5 hours at 37°C in a shaker at 75 rpm. (Yeast adheres to the surface of the wells).

Step 2

Cell suspension was aspirated and wells washed twice with 150 ml of Phosphate - Buffered Saline (PBS) to remove closely adherent cells.

Step 3

100 ml of yeast Nitrogen base medium was transferred to the wells and Incubated at 37°C (75 rpm)

Step 4

Biofilm was allowed to develop for up to 66 hours. Yeasts were quantified by crystal violet staining methods. The medium was replenished daily by aspiration of fresh medium.

Crystal violet staining

Biofilm formation was quantified by a modification of crystal violet assay. Biofilm plates were washed twice with 200 ml of PBS, air dried for

45 minutes. Wells were stained with 110 ml of 0.4% aqueous crystal violet solution for 45 minutes. *Candida albicans* ATCC 90028 and *Candida parapsilosis* ATCC 96142 were used as controls.

Antifungal susceptibility test

Anti-fungal susceptibility testing was performed on Muller Hinton agar with 0.5 µg/ml methylene blue and 20% glucose. The turbidity of the inoculums was compared with 0.5 McFarland standard. Tests were interpreted after 24 hours incubation at 37°C by visual method with reading mirror and compared with controls (*C. albicans* ATCC 90028 and *C. parapsilosis* ATCC 95142). The zone size was compared with NCCLSI guidelines.

RESULTS

A descriptive study was carried out in a tertiary care centre, Chennai for a period of six months. Both in- patients and out patients samples

were included in this study. A total of 68 *Candida* species were isolated and included in this study. In our study we observed Candidial infections were more among the in-patients 69.1% (n=47) than out patients 30.8% (n=21) with an increased number of female predominance n= 39 (57.3%) than male patients n= 29(42.6%). The maximum number of isolates were obtained from patients in the age group 31-50 years. The demographic distribution of the patients included in the study is shown in table 1; fig 1

The mentioned table 2 shows the commonest source of isolation of *Candida* species in the decreasing order of frequency with increase number in urine samples 70.5% (among which non –catheterised sample were 68.7%) followed by exudates 20.5%, respiratory 5.8% and blood 2.9%.

Identification of *Candida* species

On performing germ tube test we found that *C. albicans* were n=46 (67.6%) and *Non-albicans Candida* n= 22 (32.3%). Based on germ

Table 1. Demographic distribution of patients

Age	10-20	21-30	31-40	41-50	>50
Male	3	6	9	6	5
Female	2	2	11	18	6
TOTAL	5(7.3%)	8(11.7%)	20(29.4%)	24(32.3%)	11(16.1%)

Table 2. Isolation of *C. albicans* and *Non – Albicans Candida* from various clinical samples

Sample	Total number	<i>C. albicans</i>	<i>Non Albicans Candida</i>
Urine (voided and catheter)	48(70.5%)	29(42.6%)	17(27.9%)
Exudate	14(20.5%)	11(16.1%)	5. (4.4%)
Respiratory	4 (5.8%)	4 (5.8%)	-
Blood	2 (2.9%)	2 (2.9%)	-

Table 3. Biofilm formation by *Candida* species

Biofilm formation	<i>C. albicans</i>	<i>C. tropicalis</i>	<i>C. glabrata</i>	<i>C. parapsilosis</i>
Heavy	26	1	3	2
Moderate	10	11	3	2

tube test and chlamydoconidia production tests, CHROM agar test and sugar assimilation tests were performed. In our study among the NAC, *C. tropicalis* ranks the first n= 12(54.5%), n= 6 (27.2%) were *C. parapsilosis* and only n= 4 (18.1%) of *C. glabrata*; fig 3, fig 4, fig 5

Biofilm formation was done by using 96 microtitre plate method and quantified using 0.4% crystal violet. *C. albicans* showed heavy biofilm formation, *C. tropicalis* and *C. glabrata* showed moderate biofilm formation and other *Candida* species showed weak biofilm formation. Table 3; fig 6, fig 7

Antifungal susceptibility pattern of the *Candida* isolates is shown in below table 4, table 5

C. parapsilosis showed maximum sensitivity to fluconazole (75%) followed by *C. albicans* (69.5%) and *C. tropicalis* (58.3%). All flucytosine resistant *Candida* strains were found

to fluconazole resistant. Keeping in mind the intrinsic resistance to azoles by *C. glabrata*. MIC for the resistant strains n=20 shown in the below table 6

Fluconazole exhibited MIC 50 at 0.50µg/ml for *C. albicans*, 1.00 µg/ml for *C. tropicalis* and *C. parapsilosis* 0.50 µg/ml. Flucytosine showed MIC 50 at 0.19µg/ml for *C. albicans* and *C. tropicalis* at 0.047µg/ml for *C. parapsilosis* at 0.032µg/ml. Amphotericin B exhibited MIC 50 at 0.016µg/ml for *C. albicans*, 0.002µg/ml for *C. tropicalis* and *C. parapsilosis* >0.002µg/ml.

Fluconazole exhibited MIC 90 at 64.0µg/ml for *C. albicans*, 2.00 µg/ml for *C. tropicalis* and *C. parapsilosis* 1.00 µg/ml. Flucytosine showed MIC 90 at 0.75µg/ml for *C. albicans* and *C. tropicalis* at 0.125µg/ml for *C. parapsilosis* at 0.047µg/ml. Amphotericin B exhibited MIC 90 at >0.002µg/ml for *C. albicans*, >0.002µg/ml for *C. tropicalis* and *C. parapsilosis* >0.002µg/ml. In this study candida

Table 4. Fluconazole susceptibility testing of *Candida* isolates

Fluconazole	<i>C. albicans</i> n=46	<i>C. tropicalis</i> n=12	<i>C. glabrata</i> n=6	<i>C. parapsilosis</i> n=4
Sensitive	32(69.5%)	7(58.3%)	-	3(75%)
Resistant	14(30.4%)	5(41.6%)	6(100%)	1(25%)

Table 5. Flucytosine susceptibility testing of *Candida* isolates

Flucytosine	<i>C. albicans</i> n=46	<i>C. tropicalis</i> n=12	<i>C. glabrata</i> n=6	<i>C. parapsilosis</i> n=4
Sensitive	38(82.6%)	9(75%)	6(100%)	4(100%)
Resistant	8(17.3%)	3(25%)	-	-

Table 6. Minimum inhibitory concentration of resistant strains

Species	Antifungal Agent	50s	90s	Range
<i>C. albicans</i>	Flu	0.50	64.0	0.064-<256
	Fe	0.19	0.75	0.023-2
	Amp	0.016	0.012	>0.002-0.064
<i>C. tropicalis</i>	Flu	1.00	2.00	0.5-2
	Fe	0.047	0.125	0.023-0.19
	Amp	0.002	0.016	>0.002-0.047
<i>C. parapsilosis</i>	Flu	0.50	1.00	>0.25-1
	Fe	0.032	0.047	0.003-0.5
	Amp	>0.002	>0.002	>0.002-0.002

Flu- Fluconazole , Fe- Flucytosine , Amp- Amphotericin B

isolates showed 83% susceptibility to Fluconazole. For Flucytosine, read at almost complete inhibition of growth (98%) and for amphotericin B, at the point of complete inhibition (100%).

DISCUSSION

The last few decades have seen an increase in Candidemia.⁽¹²⁾ Of all the known species of *Candida* only 10% are pathogenic to humans. Unlike the previous reports we are now universally noticing an increase in the incidence of NAC.

A total of 68 *Candida* species were isolated over a period of six months from various clinical samples, in a tertiary care centre, Chennai. Out of 68 *Candida* species, 46(67.6%) were *C.albicans*, the rest were NAC of which 12(17.6%) *C.tropicalis*, 6(8.8%) *C.parapsilosis* and 4(5.8%) *C.glabrata*. Among the isolates, *C.albicans* predominated i.e (67.6%). Predominance of *C.albicans* were also observed in other studies

done by L.Sumithradevi *etal*⁽¹³⁾ which showed 31 (52%) *C.albicans* isolated out of 60 samples, and in another study done by Sri Janani B *et al*⁽¹⁴⁾ 27 (39%) *C.albicans* were isolated out of 70 samples.

However higher incidence of NAC were also observed in various studies. In this study among the non albicans species, *C.tropicalis* is found to be the most predominant species similar to the study done by Kothavade RJ *et al*⁽¹⁵⁾ out of 429 samples 64% were *C.tropicalis* and only 14% were *C.albicans*. The reason for predominance or rise in infections of *C.tropicalis* or NAC is unknown.

Given that females are more susceptible to *Candida* infection due to physiological factors (age, pregnancy, use of oral contraceptives) could be the reason why our study shows *Candida* species isolated were more in the female patients 39 (57.3%) similar to the study done by Dr.Jayalakshmi L *et al*⁽²⁾ which showed that out of 100 samples 57 *Candida* species were isolated from female patients.

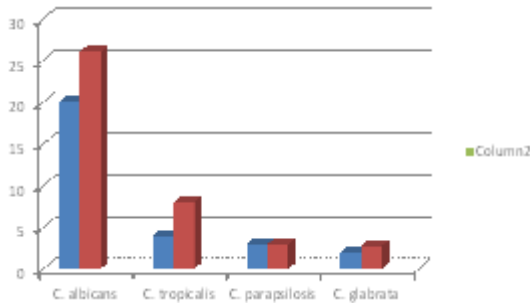


Fig. 1. Distribution of *Candida* species in male and female

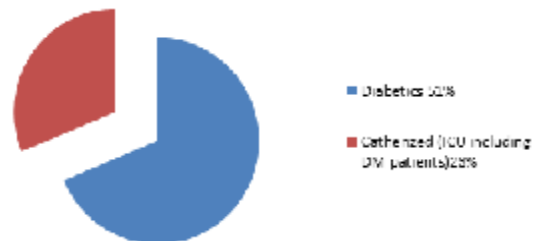


Fig.2. Immune status of our study subjects



Fig. 3. CHROM agar identification of *Candida* species

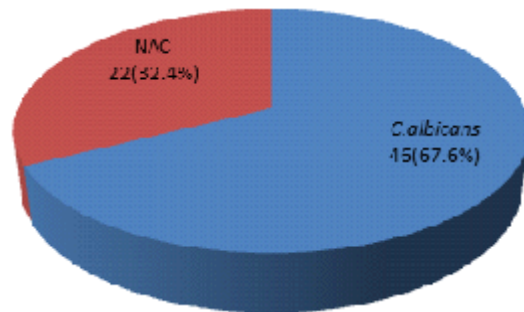


Fig. 4. Break up of *Candida* isolates



Fig. 5. Break up of Non Albicans Candida species

The sample gaining importance is urine due to increased incidence of biofilm production in the Catheterised patients. Biofilm as a virulence factor thus appears to contribute most in pathogenesis of urinary tract infection and other luminal infections, compared to other clinical conditions. In this study increased number of *Candida* were isolated from urine samples ; non – catheterised samples 33(68.7%) which is similar to a study done by N Pahwa *et al* ⁽¹⁶⁾ were of 237 samples the maximum isolates were from urine samples 19% were from voided sample and (13.9%) from urine of patients with in-dwelling urinary catheter .A study by Tumbarello *et al* ⁽¹⁷⁾ showed .*C. albicans* was predominant in catheterized patients than non albicans out of 294 patients 136 were positive for *C. albicans* and 90 were positive for non albicans unlike our study were we found that out of the 15 (23%) samples from catheterized patients 6 were positive for *C. albicans* and 9 were positive for non albicans.

In our study CHROM agar was helpful for presumptive identification of NAC and can be used in case of multi species opportunistic infection. In our study out of 68 isolates, culture based on CHROM agar showed 100% growth accuracy to *C. albicans*, *C. tropicalis*, *C. parapsilosis* and *C. glabrata*, similar to the studies done by Barry AL, Brown SD *et al* ⁽¹⁸⁾ and Beighton.D *et al* ⁽¹⁹⁾. Another study done by Jain N *et al* ⁽²⁰⁾ reported that the performance of a commercially available chromogenic *Candida* speciation as highly accurate out of 429 samples processed on CHROM agar showed over 82% accuracy to all the isolates.

The biofilm growth protects the microorganism from the host defense and antimicrobial agents. In this line, biofilm formation

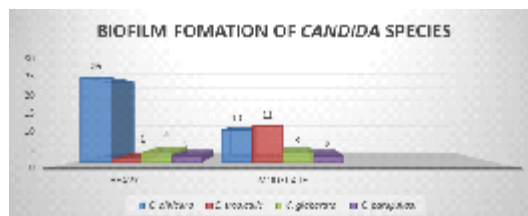


Fig. 6. Biofilm formation of *Candida* species

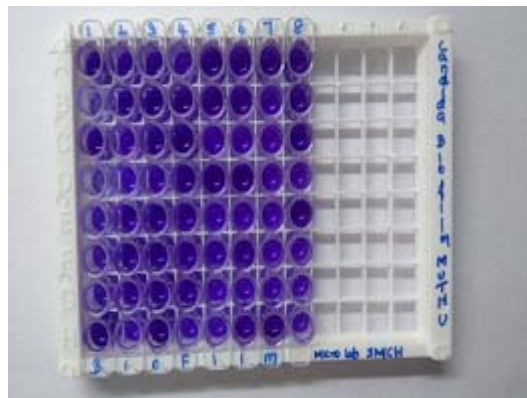


Fig.7. Biofilm production of *Candida* species

is a risk factor that increases the mortality rate in Candidiasis in critically ill patients or immunocompromised individuals.⁽¹⁷⁾ Recent studies have documented a shift toward NAC species from *C. albicans*.⁽²¹⁾ Some studies have reported increasing trend of incidences of infections caused by NACs, gradually surpassing *C. albicans* as cause of Candidemia in some regions.⁽²²⁾ Factors such as increased use of antifungal drugs and broad spectrum antibiotics, long-term use of catheters and increase in the number of immunocompromised patients have contributed to the emergence of NAC species in increasing numbers.^(15,23-24) In this study *C. albicans* isolates showed strong biofilm formation similar to the study done by Ana siladias *et al* ⁽²⁵⁾ which showed out of 184 isolates 49 *C. albicans* showed heavy biofilm formation. Although in our study the percentage of strong biofilm producers of NAC is not very high, this finding could be incidental and cannot be generalized as the sample size is very small and needs to be studied in higher number of isolates.

Biofilms represent the most prevalent type of microbial growth in nature and are crucial to the development of clinical infections.⁽²⁶⁾ The ability

to form extensive biofilms on the surface of catheters, and other prosthetic devices also contributes to the high prevalence of the organism as etiologic agent of intravascular nosocomial infections.⁽²⁷⁾ Biofilm formation should be considered as an important virulence determinant during candidiasis.⁽⁵⁾

Diabetes mellitus is reported to be a general risk factor for *Candida* infections, as the glucose is thought to serve as the carbohydrate energy source required by *Candida* for biofilm formation, perhaps necessary to produce the polysaccharide matrix. Hence, it is plausible that a hyperglycaemic condition may favour adaptation of *Candida* organisms to a biofilm lifestyle. In this study out of 68 patients 33 (48.5%) were diabetic related to the study done by Tumbarello *et.al*⁽¹⁷⁾

In this study antifungal susceptibility test for fluconazole showed 69.5% of *C. albicans* as sensitive and 30.4% as resistant to the drug which is similar to the study done by Dr.Jayalakshmi *et.al*⁽²⁾ which showed (n=25) 84.8% sensitivity and (n=4) 12.1% resistance. The same study shows (n=15) 28.6% sensitivity to *C.tropicalis* and (n=18) 64.3% resistance. In our study *C. tropicalis* showed 58.3% sensitivity (n=7) and 41.6% resistance (n=5). *C. Parapsilosis* showed (n=3) 75.5% sensitivity and (n=1) 25% resistance related to the above mentioned study which showed (n=7) 63.6% sensitive and (n=3) 27.3% resistance. In our study antifungal susceptibility test for flucytosine showed *C.albicans* n=8 (17.3%), *C.tropicalis* n=3 (10.86%). No resistance observed among *C.glabrata* and *C.parapsilosis* similar to study done by N Pahwa *et al*⁽¹⁶⁾. Antifungal susceptibility test for Amphotericin B showed 100% sensitivity.

In our study resistance strains n=20 were selected and MIC was done using E-STRIP for fluconazole, Amphotericin B, Flucytosin. In present study Candidial isolates showed 69.5% susceptible to Fluconazole similar to the study done by R Adhikary *et al*⁽²⁸⁾ which showed 75% susceptibility to Fluconazole. In this study for flucytosine, read at almost complete inhibition of growth (98%) and for amphotericin B, at the point of complete inhibition (100%) similar to the study done by Sharon C.A.Chen *et.al*⁽²⁹⁾ which showed (90- 94%) susceptible to flucytosin and (100%) susceptible to Amphotericin B.

CONCLUSION

Due to the increasing incidence of *Candida* infections, there is great interest in *Candida* virulence factors, which are in turn important in the establishment of the strategies for control and prevention of Candidiasis.⁽³⁰⁾ NAC species cannot be overlooked as mere contaminant or nonpathogenic commensals. Research on prevalent *Candida* species along with their virulence factors in a given set up would be an important tool to prove the relation between the infective species of *Candida* and infection. The changing patterns of the *Candida* isolation from various clinical samples have made identification of *Candida* species producing virulence factors compulsory for diagnostic Microbiology service.⁽³¹⁾ This changing trend of causative role of *Candida* in different studies from different parts of the world and from India and the emergence of NAC species and their association with virulence factors cannot be overlooked.⁽²³⁾ Increased isolation and complete identification of *Candida* species in more Microbiology laboratories might be instrumental in reports of rising NAC emergence. More multicenteric studies on larger sample size will definitely go a long way in revealing epidemiology, emergence and spread of NAC.

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