

## Pathogenicity Testing of Clinical *Candida* Isolates by Assessing Biofilm Formation and their Adhesion to Urinary Catheter Material

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**Candida is one of the most common microorganisms forming biofilms. The present study was aimed to evaluate biofilm formation in different clinical Candida isolates and their adhesion to urinary catheter materials. The study comprised 150 Candida isolates from clinical samples. Colonies of Candida were identified to species level using standard tests. Biofilm formation was studied on microtitre plates. Adhesion assay for the biofilm producers was performed on urinary catheter. Statistical Analysis was done by Chi square test. From 150 isolates collected; highest number of candida isolates were recovered from blood culture (44%) followed by urine (22.7%). Though *C. albicans* was the commonest isolate (44%), *C. krusei* was the most common species isolated from blood cultures (42 of 66; 63.6%). A total of 41 *Candida* isolates were found to produce Biofilm (27.3%; 41/150). The proportion of the biofilm producers in blood (27.3%), urine (32.4%) and exudates (29.6%) was almost identical. Lowest proportion of biofilm producers was found on dentures (17.4%) (Non-significant; p value 0.53). Higher biofilm producing tendency in urinary isolates may be contributory to their potential to cause UTI in catheterized patients. Higher isolation of *C. krusei* from blood samples was a noteworthy finding.**

**Keywords:** Candida, Biofilm, adhesion, urinary catheter.

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Biofilms are structured microbial communities attached to surfaces. They secrete exopolymeric material often referred to as matrix<sup>1</sup>. *Candida* species are one of the most common fungi producing biofilms. Ability of candida to produce hyphal structures provides architectural integrity to the biofilm. The structure of biofilm helps candida to survive harsh environmental conditions. Once lodged, *Candida* cells are capable of forming matured biofilms in 20 hrs<sup>2</sup>. Surfaces of implants, temporary intravascular devices or removable aids

like denture frequently provide suitable surfaces for biofilm formation. Biofilms are difficult to dislodge. They are the continuous source of infection leading to disseminated infections or septicemia<sup>3</sup>. The fungal biofilms are becoming irksome clinical and economical problem with higher mortality rates. Infections due to biofilm forming *candida* are difficult to manage and the hospital stay in these cases is usually prolonged<sup>4</sup>.

Of late, the non-albicans candida (NAC) are frequently reported causing clinical infections and a number of these isolates are shown to produce biofilms. In fact, some workers have reported higher biofilm production in NAC isolates from blood stream infections.

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### Aim and Objectives

The present study was aimed at testing for two important factors influencing pathogenicity of candida isolates, i.e., identification of biofilm producers and adhesion to urinary catheter materials, with the following objectives.

1. Isolation and speciation of *Candida* isolates from clinical specimens.
2. Detection of biofilm producers among the clinical *Candida* isolates.
3. Evaluate adhesion of *Candida* isolates to urinary catheter material.
4. Comparison of the biofilm formation in both *C. albicans* and NAC spp. isolated from clinical specimens.

### MATERIAL AND METHODS

The study was begun after obtaining permission from the institutional ethical committee. We included 150 *Candida* species isolated from clinical samples received at Clinical Microbiology laboratory of our hospital, from November 2014 to November 2015. The *Candida* isolates were identified by standard methods including Gram's stain, germ tube test, chlamyospore formation; carbohydrate assimilation and temperature sensitivity test<sup>5</sup>. The speciated candida isolates were stored on Sabouraud's Dextrose Agar (SDA) slants at 4 °C for further study. The identified *Candida* isolates were subjected to biofilm formation and adhesion tests. The pure isolates were subcultured on SDA (Hi Media, Mumbai) and a single colony was transferred to tubes having Sabouraud's Dextrose broth. The tubes were incubated in a shaking incubator at 37 °C at 120 rpm for 18 hrs. Cells were harvested by centrifugation and washed thrice with sterile phosphate-buffered saline (PBS, pH: 7.2). The yeast cells were re-suspended in fresh buffer and standardized to a concentration of  $1 \times 10^7$  to  $1 \times 10^6$  cells/mL. Estimation of Biofilm formation in different *Candida* species.

Biofilm formation was studied on sterile, polystyrene; flat-bottom 96-well microtitre plates (Laxbro, India) using the method explained by Taff *et al.*, (2011)<sup>6</sup>.

#### Incubation of *Candida* for biofilm formation

Each well of the microtitre plate was filled with 100 µl of a standardized cell suspension

( $10^7$  cells/ml). The plate was incubated for 1.5 hours at 37 °C at 75 rpm to allow the yeast cells adhere to microtitre wells. A known biofilm producing *Candida albicans* isolate and a known biofilm negative *Candida albicans* isolate from our laboratory were used as positive and negative controls. With each test run, 100 µl of PBS was added to a well and 100 µl of SD broth were used as blank and media control respectively. After the incubation, the supernatant was carefully removed and the wells were gently washed two times with 200 µl of PBS to remove unbound cells. The washed wells were filled with 100 µL SD broth, and the plates were incubated at 37°C in a Shaking incubator at 75 rpm. The SD broth was replaced with fresh medium two times a day and incubation was continued up to 48 hours. The isolates were tested in triplicate. All the inoculations and media replacements were done in a bio-safety cabinet Class 2 A 2.

#### Estimation of biofilm development by Crystal violet assay

After the completion of incubation the wells were washed twice with 200 µL of PBS and allowed to dry at room temperature for 45 minutes. The washed wells were stained with 100 µL of 0.4% crystal violet solution for 45 minutes. Each well was washed manually four times with 350 µL of sterile distilled water to remove excess crystal violet. For de-staining 200 µL of 95% ethanol was added to the wells and plates were kept at room temperature for 45 minutes. 100 µL of solution from the wells was transferred to wells in a new microtitre plate. The optical density was measured at 600 nm (Tecan, Sunrise, Austria).

#### Adhesion assay on the urinary catheter

Adhesion assays were performed using a modification of the technique described by Uzunoglu *et al.*, (2014)<sup>7</sup>. Briefly, urinary catheter materials (PVC, RUCH, Germany) were cut to get several 1 cm pieces using sterile surgical blades inside the biosafety cabinet. The pieces were put in a Petri plate. The standardized *C. albicans* cell suspension in PBS ( $1 \times 10^6$  cells/mL) was added to each Petri plate to cover the pieces and incubated for 2 hours at 37 °C to facilitate adhesion. After incubation, the pieces were gently washed twice with 5 mL PBS to remove the unadhered cells.

#### Counting of adhered *Candida* cells

Washed catheter pieces were put in a

sterile test tube consisting 5 ml normal saline and then were pulse agitated using a vortex mixer at the maximum speed for 30 seconds and left at room temperature for 5 minutes and 100 µl of the suspension was spread uniformly on SDA plates. The plates were incubated at 37°C for 24 hours and colonies were counted. The colony forming units (CFU) per millilitre for candida were calculated.

All the 41 biofilm producing candida isolates and 40 non-biofilm producers candida isolates were studied for adhesion to the urinary catheter material.

#### Statistical Analysis

Chi square test was used for statistical analysis to know the significance of test results.

## RESULTS

#### Isolation of candida from clinical specimens with their speciation

A total of 150 clinical candida isolates were recovered during in the study period. Table 1., shows sample wise distribution of the isolates. Of the 150 isolates 66 (44%) were *C. albicans* and 84 (56%) were NAC. Highest number of isolates were obtained from blood samples (44%) followed by urine samples (22.7%). Though overall isolation of NAC was higher than *C. albicans*; in all the samples except from blood, *C. albicans* isolation was higher compared to NAC. *C. krusei* (37.3%) was the commonest isolate among the NAC group and also from each sample type. The isolation of *C. krusei* from blood (36.7%), however, outnumbered the other sample groups. Other NAC species obtained were *C. tropicalis*, *C. guilliermondii*, *C. glabrata*, *C. parapsilosis*, *C. lusitaniae*, *C. kefyr* and *C. dubliniensis*.

Majority of the isolates in the present study were from blood (66/150) followed by 34 from urine, 27 from exudates and 23 from dentures. *C. krusei* was the commonest isolate from blood. Out of the 66 blood isolates 42 were *C. krusei* while *C. albicans* were 11. *C. albicans* was the commonest isolate from urine, exudates and dentures. *C. krusei* was the second common isolate in each sample type. A comparison of proportion of *C. albicans* and NAC isolation was done by Chi<sup>2</sup> test. For blood the difference in the proportion was highly significant (Chi square is 24.79,  $p < 0.001$ ).

#### Biofilm production

The biofilm production by all the 150 isolates was assessed by the method described by Taff *et al.* (2011) <sup>6</sup>. As shown in Table 2. among total candida isolates, 41 (26.7%) showed biofilm production. Of 66 *C. albicans* isolates 20 produced biofilm (30.3%) as against 21 of the 84 (25%) NAC showing biofilm production. *C. krusei* (12/55; 21.8%) was the highest biofilm producer among the NAC isolates. *C. dubliniensis*, *C. glabrata* and *C. kefyr* did not show biofilm production. *C. albicans* was the commonest biofilm producer from all the samples except blood. In urinary biofilm producers 7 of 11, from exudates 7 of 8 and from dentures, 3 of 4 were *C. albicans*. Thus, except for blood *C. albicans* outnumbered NAC in the biofilm producers. In blood, of 18 biofilm producers 10 were *C. krusei*.

The proportion of the biofilm producers in urine (11/34; 32.4%) and exudates (8/27; 29.6%) was almost identical followed by 27.3% blood isolates showing biofilm production. Lowest proportion of biofilm producers was found on dentures (17.4%). *C. albicans* isolates from exudates were more frequent biofilm producers (7/18; 38.9%) while for blood, *C. krusei* were more common in biofilm production.

On statistical analysis (Chi<sup>2</sup> test), there was no significant difference in percentage of biofilm producers among different clinical specimens ( $p = 0.28$ ).

#### Adhesion pattern in biofilm producing candida isolates

All the 41 biofilm producing isolates were subjected to adhesion study <sup>7</sup>. The details are shown in Table 3, Graph 1. Both *C. albicans* and NAC showed adherence to urinary catheter material, the *C. albicans* isolates gave higher CFUs compared to NAC isolates. Species and sample wise prevalence of adhesion is shown in Table 3. There were no quantitative differences among different species on comparison of their CFUs except two *C. albicans* isolates from dentures showing strong adherence. Mean adhesion in all different clinical isolates was shown to be significant ( $p = 0.039$ ) by Kruskal Walli's test. However mean adhesion between the clinical groups compared by Mann-Whitney test was non significant.

### Adhesion pattern in non-biofilm producing candida isolates

The results were further compared with the adhesion pattern of non-biofilm producing candida isolates. A total of 40 non-biofilm isolates were subjected to adhesion test (table 3). Only 6 of the 40 isolates showed adherence. Association of adherence to biofilm production in biofilm positive and biofilm negative isolates showed very high significance p value < 0.00000.

### DISCUSSION

*Candida* infections are gaining a lot of importance of late. *Candida albicans* was, so far, considered to be the pathogenic species and there was a tendency to neglect the non albicans isolates (NAC). The scenario is changing fast. Influenced by several predisposing factors like use of immunosuppressive agents, antibiotics and long term hospitalization etc. *Candida* infections

**Table 1.** Isolation of candida species from various clinical isolates biofilm producing and non biofilm producing

	No. of isolates	NON CANDIDA ALBICANS SPECIES n (%)										
		<i>C. albicans</i> (n(%))	NAC species(n(%))	<i>C. albicans</i>	<i>C. krusei</i>	<i>C. parapsilosis</i>	<i>C. tropicalis</i>	<i>C. guilliermondii</i>	<i>C. lusitaniae</i>	<i>C. kefyfyr</i>	<i>C. glabrata</i>	<i>C. dubliniensis</i>
Blood	66	11 (16.6)	55 (83.3)	11 (16.6)	42 (63.6)	2 (3.0)	4 (6.0)	3 (4.54)	1 (1.5)	1 (1.5)	2 (3.03)	0
Exudate	34	18 (52.9)	9 (26.4)	18 (52.9)	6 (17.6)	0	2 (5.8)	0	0	0	0	1 (2.94)
Urine	27	25 (92.5)	9 (33.3)	25 (92.5)	2 (7.4)	1 (3.7)	2 (7.4)	1 (3.7)	1 (3.7)	1 (3.7)	1 (3.7)	0
Dentures	23	12 (52.1)	11 (47.8)	12 (52.1)	5 (21.7)	0	0	3 (13.0)	1 (4.3)	0	2 (8.69)	0
	150	66	84	66	55	3	8	7	3	2	5	1

**Table 2.** Biofilm producers among the clinical isolates

Candida group	Species	BLO OD	EXUDA TE	URI NE	DENTU RES	TOT AL
<i>C. albicans</i>	<i>C. albicans</i>	2	7	7	3	19
Non Albicans <i>Candida</i>	<i>C. krusei</i>	10	1	1	0	12
	<i>C. parapsilosis</i>	2	0	1	0	3
	<i>C. tropicalis</i>	2	0	1	0	3
	<i>C. guilliermondii</i>	0	0	1	0	2
	<i>C. lusitaniae</i>	1	0	0	1	2
	<i>C. kefyfyr</i>	1	0	0	0	1
	<i>C. glabrata</i>	0	0	0	0	0
	<i>C. dubliniensis</i>	0	0	0	0	0
		18	8	11	4	41

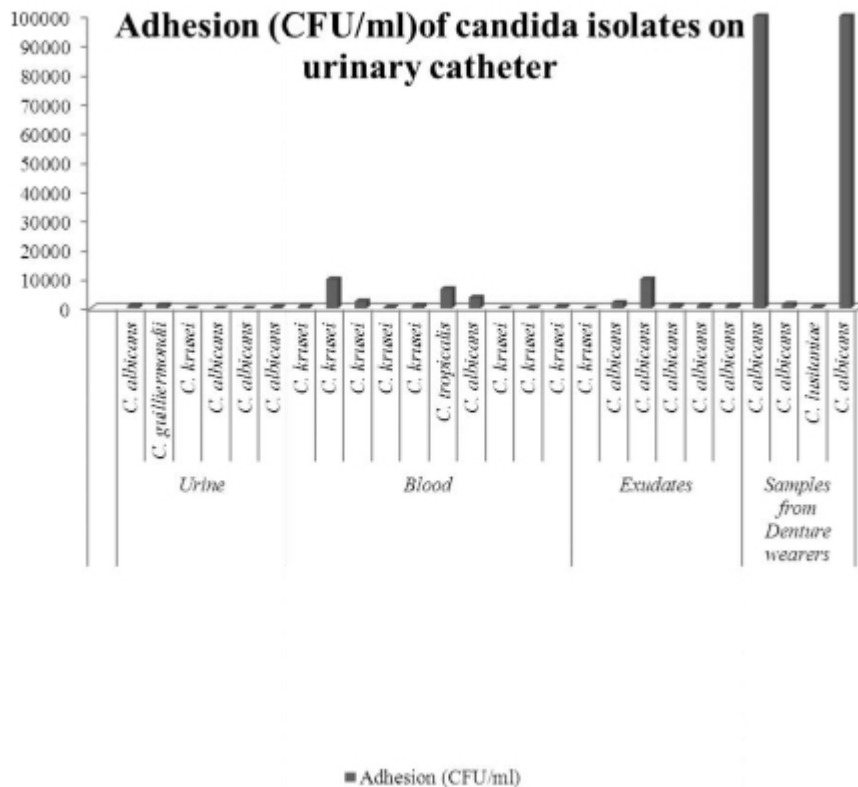
have become frequent <sup>8,9,10</sup>. Among the virulence factors, biofilm production is considered as an important virulence attribute <sup>11</sup>. The present study was conducted to speciate candida isolates from clinical specimens and to study their adherence to urinary catheter material and also to test the isolates for biofilm production. The study was conducted at Department of Microbiology, SDM College of Medical Sciences and Hospital, Dharwad from November 2014 to November 2015. A total of 150 candida isolates from blood, urine, exudates and

denture surfaces were speciated (Table 1). NAC (56%) outnumbered *C. albicans* (44%) and a total of 8 different NAC species were encountered in this work. *C. krusei* was the commonest NAC isolate and was the predominant isolate from blood. Except *C. krusei*, all the other seven isolates tallied in single figure.

*Candida* isolates from blood should not be neglected and dismissed as contaminants <sup>12</sup>. Frequent isolation of *C. krusei*, especially from neonatal samples, was alarming. We, therefore, did environmental sampling of neonatology unit. Samples from hands of neonatology staff were also collected (Data not shown). However, none of these samples yielded candida species. Vinita and Mamata (2011), showed *C. krusei* as the most prevalent NAC isolate (60.36%) <sup>10</sup>. Similarly studies from Mujika *et al.*, 2004 showed prevalence of *C. krusei* from respiratory tract samples <sup>13</sup>. *C. krusei*, though less virulent, is recognized for its fluconazole resistance <sup>14</sup>. In all the samples other than blood *C. albicans* were proportionally higher

**Table 3.** Adhesion pattern in biofilm producing candida isolates

	Biofilm positive isolates	Biofilm negative isolates
	41	40
Adherence seen	37	6
No adherence seen	4	34



**Graph 1.** Adhesion pattern in biofilm producing candida isolates



than NAC. Except for blood the comparison of proportion of *C. albicans* and NAC was non significant for all the samples. For blood the difference was highly significant ( $p < 0.006$ ).

We observed high candida isolation from newborns rather than from other age groups. Wadile and Bhate (2015) showed isolation of *candida* in 32.26% of neonatal septicemia cases among which NAC accounted for 35%<sup>15</sup>. Juyal *et al.* (2013) found 80.30% NAC species in neonatal candidemia, where *C. parapsilosis* (25.0%) and *C. tropicalis* (21.97%) were the most predominant species<sup>16</sup>. The prevalence of NAC species could be dependent upon geographical area and type of clinical specimen. During our previous work we had speciated 534 candida isolates from oral cavity and found *C. guilliermondii* as the second common isolate<sup>17</sup>. Geographical variations in the distribution of candida species have been described. It is also reported that a species preponderant in a geographical area may subsequently be replaced by other species<sup>2</sup>. The increased number of NAC isolation and high prevalence of *C. krusei* in the present study suggests the need for speciation of clinical candida isolates.

All the isolates were tested for biofilm production by tissue culture plate method (TCP). Biofilm producing isolates were marginally higher in *C. albicans* group. Out of 42 *C. krusei* from blood 10 showed biofilm production. Biofilm producing ability is an important virulence attributes. Biofilm formation is known to be the important virulence factor in the infection process<sup>18</sup>. Among the 150 candida isolates of our study 41 were positive for biofilm production (26.7%). In the studies done by Mohandas and Ballal<sup>19</sup>, 2007 and 2011 they showed 50 and 60% of the isolates being biofilm producers respectively<sup>10</sup>. Udayalaxmi *et al.*, (2014), showed that 70% of the isolates produced biofilm<sup>20</sup>. In our study, the proportion of the biofilm producers in urine and exudates was almost identical (urine – 29.4% and exudates – 29.6%) followed by blood giving 27.3% biofilm producers. Lowest proportion of biofilm producers was found on dentures (17.4%). The exudates samples obtained were commonly from in association with various intravenous tubes like endotracheal tubes etc. Similarly a few studies claim that some isolates are more prone to adhere and form biofilm depending on their isolation

site (Shin *et al.*, 2002; Hasan *et al.*, 2009; Silva *et al.*, 2010a, 2011; Mohandas and Ballal, 2011). In a study done at Manipal, India by Mohandas and Ballal (2011), *C. albicans* isolates recovered from blood exhibited lower percentage of biofilm positivity. In the study of done by Udayalaxmi *et al.*, (2014), biofilm production was more in candida isolated from urine samples in comparison to vaginal isolates<sup>20</sup>.

Except for 5 samples from dentures none of the other samples gave mixed candida growth.

The samples, isolates and their biofilm forming ability were statistically analyzed. However, there was no significant relationship between these factors.

Adhesion is an important attributes of pathogenicity of candida. Adhesion facilitates colonization which is the initial step for most pathogens proceeding to clinical infections. The materials of artificial devices could be critical factor in deciding the success of colonization. Adhesion on the surface is the initial event in Biofilm formation<sup>22</sup>. However, the materials of artificial devices could be critical factor in deciding the colonization<sup>23</sup>. We, therefore, studied adhesion in all the 41 biofilm producers recovered in the study. We also subjected 40 biofilm negative isolates with specimen-wise matching numbers for adhesion.

Urinary catheters are one of the most common devices related to candida infections<sup>24,25</sup>. As this is a routine device used in the management of various conditions, we chose urinary catheter material to study adherence. The catheters used were made of PVC, RUCH, Germany. Of the 41 biofilm producers 37 isolates showed adherence where CFUs ranged from 20 to 10<sup>5</sup> per ml. The highest number of CFU of 10<sup>5</sup>, was seen in two *C. albicans* isolates from dentures. The maximum CFUs yielded by biofilm producers were 10<sup>5</sup>/ml whereas for non biofilm producers the maximum CFU number was only 10<sup>2</sup>/ml. Only six of the 40 non biofilm producers tested showed adherence.

Though adhesion is facilitated by biofilm production, several other factors including zeta potential, contact angle, mucin, other bacteria in the niche and hydrophobicity play important role in adhesion<sup>26,18</sup>. Studies by Tamura and Gasparetto (2003) showed adherence of *C. albicans* was significantly greater than *C. parapsilosis* on latex,

but it was comparable on silicon<sup>23</sup>. Catheter related candidemia, age related candida infections, etc. are increasing in recent years<sup>1, 8, 9, 10, 13</sup>. Literature suggests that, almost invariably, an implanted device such as an intravascular or urinary catheter or endotracheal tube, is associated with candida infections and a biofilm can be detected on the surface of these device<sup>20, 24, 25</sup>. Present study didn't reveal any significant association between in biofilm production of the isolates and the clinical samples from which they were coming. Though, adherence to the urinary catheter and biofilm forming ability significantly co-existed, there was no significant difference in adherence between the different candida species observed.

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