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Biofilm production by clinically isolated *Candida*: Comparative analysis based on specimen, methodology, and various *Candida* species

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ABSTRACT

Aim and objectives: The importance of *Candida* as a human pathogen has increased, particularly in the hospitalized and immune compromised patients. *Candida albicans*, the predominant species worldwide, is now being replaced by the more resistant non-*albicans Candida*. This study compares various biofilm producing clinical isolates of *Candida* species by three commonly available methods for biofilm detection.
Material and methods: Clinical samples from patients with suspected fungal/*Candida* infections were cultured. *Candida* isolates were speciated on the basis of colony morphology, germ tube formation, chlamydospore formation on corn meal agar and color production on HiCrome *Candida* agar and confirmed using Vitek-2 Yeast-ID cards. Biofilm production was performed visually by modification of the tube method, spectrophotometrically by Percent transmittance method and Crystal violet assay.
Results: 20.6% of *Candida* isolates were obtained. *Candida albicans* (33.3%) was outnumbered by the non-*albicans* species (66.7%). Crystal violet micotitre plate assay demonstrated highest positivity (54.8%), followed by percent transmittance microtitre plate method. *C. glabrata* was the highest biofilm producer, followed by *C. tropicalis.*
Conclusion: Biofilm production is very common among *Candida* isolates, more so for the non-*albicans Candida* species. Routine testing for biofilm production should be contemplated particularly in serious patients with *Candida* infection, not responding to typical anti-fungal therapy.

1. Introduction

Since 1980s, the importance of *Candida* as a human pathogen has increased, particularly in the hospitalized and immuno-compromised patients [1]. While *Candida albicans* (*C. albicans*) was the predominant species worldwide, it is now being replaced by the more resistant non-*albicans Candida*, particularly *Candida tropicalis* (*C. tropicalis*) and *Candida glabrata* (*C. glabrata*) [2]. Among several virulence factors possessed by *Candida*, biofilm plays a key role in pathophysiology of Candidiasis, particularly in relation to *in situ* prosthetic devices and catheters.

Biofilms are structured communities of microbial cells encased in a self-produced organic polymeric matrix, providing advantage in terms of nutritional availability, co-operation between cells for metabolism and acquisition of new genetic traits, as well as protection from environment and host factors. Biofilm cell communities are also known to be more resistant to antifungal drugs than the planktonic cells; with several contributing factors such as biofilm structural complexity, presence of extracellular matrix (ECM), metabolic heterogeneity intrinsic to biofilms, and biofilm-associated up-regulation of efflux pump genes [3]. Thus, infections produced by such strains are treatable with great difficulty, often responding poorly to anti-fungal drugs. Ability to form biofilm is considered a risk factor that increases the mortality rate in candidiasis in critically ill patients or immunocompromised individuals [4].

Thus, this study was designed to compare the ability of producing biofilm by various *Candida* species isolated from different clinical specimens.In addition, comparison between three commonly available methods for biofilm detection was sought for.

2. Materials and methods

This study was conducted over a period of eight months during 2015–16 at a 1600 bedded tertiary care hospital and associated medical college in New Delhi, India. Clinical samples, such as urine, blood culture, sputum, broncho-alveolar lavage, pus, biopsy material, nail, and CSF were collected from patients presenting to various

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departments of Lok Nayak Hospital, New Delhi, India with suspected fungal/*Candida* infection. Repeat samples from same patient were excluded from the study. The specimens were transported to the Mycology Laboratory, Department of Microbiology, Maulana Azad Medical College, New Delhi, India, within two hours.

The clinical material was cultured onto two Sabouraud's Dextrose Agar (SDA) slants with chloramphenicol (5 mg/l) to inhibit bacterial contamination. One tube was incubated each at 25 °C and 37 °C in ambient air and examined daily. The culture tubes were examined for fungal growth on alternate days, up to 4–6 weeks for possible slow growing molds. Blood cultures were performed onto bi-phasic Brain-Heart infusion broth and agar media; and incubated at 37 °C for 4–6 weeks; suspected *Candida*/ fungal growth was sub-cultured on SDA slants.

Smears prepared from colonies suggestive of *Candida* (creamy-white, paste-like colonies) were stained with Grams stain to look for presence of budding yeast cells. India ink wet mounts were also prepared to exclude presence of capsule. *Candida* isolates were speciated on the basis of colony morphology, germ tube formation, chlamydospore formation on corn meal agar with Tween 80 at 25 ° C and color production on HiCrome *Candida* agar (Himedia, Mumbai, India). Confirmation of *Candida* species was done using Vitek-2 Yeast-ID cards (bioMerieux, USA).

2.1. Detection of fungal biofilm

Determination of biofilm production by *Candida* isolates was performed by three independent methods: visually by modification of the tube method described by Brachini et al.;and spectrophotometrically by Percent transmittance (%T) method and Crystal violet assay [5–7]. All strains were tested in duplicate, and their averages were used to grade their biofilm producing capability. The methods are summarized below:

(i) **Modification of method described by Branchini** et al. [5]:

Sabouraud dextrose broth (SDB) with 2% glucose was further supplemented with glucose (60 g/l) to yield final glucose concentration of 8%; 9 ml of the broth was taken in each of the screw-capped conical polystyrene tubes. Next, 1 ml of 0.5 Mc Farland suspensions of yeast cells prepared in normal saline were transferred to tubes in duplicate. Incubation was done in ambient air at 36 ± 1 °C for 24 h. Subsequently, the culture broth was gently aspirated, and tubes were washed with ultrapure water (Rions India Lab Water Systems Pvt. Ltd., India) twice. The walls of tubes were stained with 1% safranin solution for 7–10 min. The adherent biofilm layer was scored visually as either negative (0), weakly positive (1+), moderately (2+ or 3+), or strongly positive (4+) as described by Pfaller et al.

(ii) **Percent transmittance (%T) method** [6]:

180 ul of SDB with 8% final glucose concentration, was taken in the wells of flat bottomed microtitre plate. 20 ul of 0.5 Mc Farland suspensions of yeast cells prepared in normal saline were added to the corresponding wells in duplicate. Two wells with 200 ul SDB with 8% glucose, served as blanks. The micro titer plate was incubated at 36 ± 1°c for 24 h with continuous shaking at 75 rpm. The plate was then washed twice with 300 u l ultrapure water per well. Subsequently, 200 u l ultrapure water was added to each well and percent transmittance was measured using spectrophotometer at wavelength of 405 nm. The %T value for each test sample was subtracted from the %T value for the reagent blank to obtain %Tbloc (amount of light blocked). Biofilm production by each isolate was scored as either negative (%Tbloc ≤ 5%), 1+ (%Tbloc = 5–20%), 2+ (%Tbloc = 20-35%), 3+ (%Tbloc = 35-50%), or 4+ (%Tbloc ≥ 50).

(iii) **Crystal violet assay** [7]:

Another microtitre plate was prepared and inoculated as described above. After washing twice with ultrapure water, microtitre plate was stained with 110 μl of 0.4% aqueous crystal violet solution for 45 min. Afterwards, each well was washed four times with 300 μl of ultrapure water and immediately destained with 200 μl of 95% ethanol for 45 min. 100 μl of destaining solution was transferred to a new well and the %T was measured with spectrophotometer at 595 nm and graded as above.

2.2. Statistical evaluation

Results were presented as proportions and percentages. 95% confidence intervals (95% CI) were calculated wherever required. Chi square test was used to compare the three methods of biofilm detection. SPSS version 23.0 was used for calculations.

For the purposes of statistical calculations and comparison, any strain positive by at least two of the three methods was considered to be producing biofilm.

3. Results

A total of 93 isolates (20.6%) of *Candida* were obtained from 451 specimens during the study period (Table 1). Majority of the specimens were derived from middle aged group individuals (21–40 years old); while there were relatively fewer very young (below 10 year age) and old (above 60 years). Male patients outnumbered the females, with male:female ratio being 1.30. One-fourth patients were from the Medicine department, while

Table 1
Details of patient population and specimen collected.

	Number (n = 451)	Percent (95% CI)
Age group:		
<10	26	5.8 (3.93–8.34)
11–20	48	10.6 (8.10–13.85)
21–30	109	24.1 (20.44–28.33)
31–40	92	20.4 (16.93–24.37)
41–50	67	14.9 (11.86–18.45)
51–60	73	16.9 (13.06–21.16)
>60	36	8.0 (5.80–11.97)
Sex:		
Male	255	56.5 (51.93–62.42)
Female	196	43.46 (38.96–49.52)
In-patient Department:		
Medicine	116	25.72 (21.90–31.36)
Dermatology	70	15.52 (12.46–19.17)
Otorhinolaryngology	54	11.97 (9.28–15.31)
Pediatrics	44	9.8 (7.33–12.86)
General Surgery	33	7.3 (5.23–10.12)
ICU	47	10.4 (7.91–13.60)
Gynecology	32	7.1 (5.04–9.87)
Others	55	12.2 (9.47–16.67)
Specimen:		
Urine	82	18.2 (14.89–23.33)
Sputum	75	16.6 (13.47–20.36)
CSF	66	14.6 (11.66–18.21)
Nail/ Skin	66	14.6 (11.66–18.21)
Biopsy	54	11.97 (9.28–15.31)
Blood culture	41	9.1 (6.75–12.12)
Broncho-alveolar lavage/ endotracheal aspirate	34	7.54 (5.42–10.38)
Miscellaneous specimens	33	7.3 (5.23–10.12)
Candida species isolated (n = 93)		
C. albicans	31	33.3 (24.56–43.43)
C. tropicalis	38	40.9 (31.42–51.03)
C. glabrata	19	20.4 (13.41–29.80)
C. parapsilosis	2	2.2 (0.12–7.97)
C. krusei	2	2.2 (0.12–7.97)
C. famata	1	1.1 (0.01–6.42)

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