

Biofilm Formation among Various *Candida* Species and its Role in Antifungal Resistance at Tertiary Care Centre, Jhalawar

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ABSTRACT

Introduction: Serious fungal infections particularly *Candida* infection have increased in recent years. It is as a consequence of increased immunosuppression associated with HIV infection, organ and tissue transplant and aggressive treatment for neoplastic and autoimmune diseases. Study aimed to investigate biofilm formation among candida species isolated from various clinical samples and its role in antifungal resistance.

Material and methods: A retrospective observational study was conducted from October 2017 to January 2019 in the Department of Microbiology, Jhalawar Medical College, Jhalawar. A total of 630 samples with suspected *Candida* infections were collected and processed. A total of 313 *Candida* isolates from various clinical samples were taken up for the study. Samples were processed by Gram staining, KOH mount and culture on SDA and BHI agar. Isolated yeasts were identified and speciated by germ tube test, chlamydo-spore formation on corn meal agar, color production on CHROM agar, sugar fermentation test and sugar assimilation test. Biofilm production was tested by Tube method and Tissue culture plate method. Antifungal susceptibility testing of isolates was performed as per CLSI guidelines.

Results: A total of 313 samples out of 630 samples were positive for candida infections. Out of 313 isolates, 157 (50.16%) were found to be biofilm producers. *Candida tropicalis* (52.86%) was most common *Candida* species to be isolated as biofilm producer followed by *C. Parapsilosis* (10.19%), *C. glabrata* (10.19%) and *C. krusei* (4.45%) while *C. albicans* was 35/157 (22.29%). Antifungal resistance was found to be more in biofilm producer and tissue culture plate method was found to be more sensitive than tube method for biofilm detection.

Conclusion: There is increasing trend of antifungal resistance among candida isolates particularly in *Non Albicans Candida* species. So, it is necessary to identify all yeast isolates up to species level and their potential for biofilm formation as it is one of the major virulence factors responsible for antifungal resistance. This will be helpful for efficient treatment, prevention of development of antifungal resistance and finally, the reduction of the treatment costs.

Key words: *Candida* Species, Biofilm, Antifungal Resistance.

immunosuppressed patients. Biofilm is one of the known virulence factors of *Candida*, an important pathogen and commensal. Microorganisms growing in a biofilm are associated with chronic and recurrent human infections and are highly resistant to antimicrobial agents. Early detection of biofilm production may be useful for clinical decision because of its suggestive property for potential pathogenic capacity of *Candida* isolates.

Biofilms are defined as microbial derived sessile communities characterized by the cells that are irreversibly attached to a substratum or to each other. They are embedded in a matrix of extracellular polymeric substances (EPS) they have produced and exhibit an altered phenotype with respect to growth rate and gene transcription. Within a biofilm, microorganisms communicate with each other by production of chemotactic particles or pheromones, a phenomenon called 'quorum sensing'. Availability of key nutrients, chemotaxis towards surface, surface adhesins and presence of surfactants are some factors which influence biofilm formation. Microorganisms growing in a biofilm are intrinsically more resistant to antimicrobial agents than planktonic cells.

High antimicrobial concentrations are required to inactivate organisms growing in a biofilm, as antimicrobial resistance can increase to 1,000-fold. With the emergence of biofilm associated diseases, there are considerable diagnostic problems for the clinical laboratory, decreased antimicrobial susceptibility, false negative cultures, visible but not cultivable organisms or inappropriate specimen. The determination of biofilm production in *Candida spp.* may be important for the management of invasive infections. There are various methods to detect biofilm production. These include

- Tissue Culture Plate (TCP),
- Tube method (TM),
- Congo Red Agar method (CRA),

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INTRODUCTION

Candida infections have emerged as important public health problems with significant morbidity and mortality. The growing number of immunocompromised individuals as a result of the HIV pandemic and the use of long-term immunosuppressive therapy in cancer and organ transplant patients have favoured the increased incidence of *Non albicans Candida* species among hospitalized and

- bioluminescent assay,
- piezoelectric sensors method and
- fluorescent microscopic examination.

Study by dag et al., 2010 reported that Tube method showed very good agreement for the isolates producing strong biofilm, whereas differentiation of isolates producing weak biofilm was difficult.¹ By the Congo red method, classification of existing biofilm was problematic. Among the three methods studied, microtiter plate method may be suggested as the most sensitive method, which is easy to conduct and applicable as a routine process.

Study aimed to investigate biofilm formation among candida species isolated from various clinical samples and its role in antifungal resistance.

MATERIAL AND METHODS

A retrospective observational study was conducted from October 2017 to Jan 2019. A total of 630 various clinical samples received in Microbiology department from patients with suspected *Candida* infection were collected and processed. The various clinical samples were including respiratory samples (sputum, bronchial wash and tracheal secretions), various body fluids, blood, urine, ear discharge, invasive devices (endotracheal tube, catheter tip and suction tip) and vaginal discharge. Samples were processed by Gram staining, KOH mount and culture on SDA and BHI agar. Isolated yeasts were identified and speciated by germ tube test, chlamyospores formation on corn meal agar, colour production on CHROM agar, sugar fermentation test and sugar assimilation test.¹ Antifungal susceptibility testing of the yeast isolates was performed by 'Disc Diffusion Method' including Amphotericin B (100 IU), Fluconazole (25µg), Nystatin (50µg), Ketoconazole (50µg), and Itraconazole (10µg) as per CLSI guidelines.²

Reference strains from quality control methods used were,

- *Candida parapsilosis* ATCC 22019
- *Candida albicans* ATCC 90028
- *Candida tropicalis* ATCC 750
- *Candida krusei* ATCC 6258

Biofilm formation ability of yeast isolates were tested by Tube Adherence Test and Tissue Culture Plate Method.³

STATISTICAL ANALYSIS

The data was statistically analysed using the statistical package for Social science (SPSS)/ 21.0 (Copyright © SPSS Inc.). Frequency of qualitative variables was calculated and correlation was tested by Chi-square test. Statistical

significance was accepted at $p < 0.05$.⁴

RESULTS

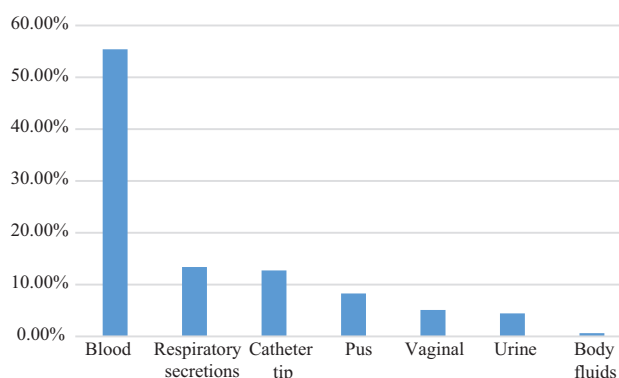
Non albicans candida 204/313 (65.18%) were predominant isolates than *C. albicans* 109/313 (34.82%). Depending on the results of various test done for speciation, *C. Tropicalis* (46.33%) was predominant isolate followed by *C. Albicans* (34.82%), *C parapsilosis* (10.54%), *C. glabrata* (5.75%) and *C. krusei* (2.56%). *C. tropicalis* was major isolate among various clinical samples whereas candida albicans was predominant. in body fluids (66.67%) and respiratory secretions (53.19%).

Tissue culture plate method (50.16%) was more sensitive than Tube method (29.07 %) for biofilm detection.

Out of 313 isolates, 157 (50.16%) were found to be biofilm producers. Maximum biofilm production was obtained in blood samples (55.41%) followed by respiratory secretion (13.38%), Catheter tip (12.74%), pus (8.28%), vaginal (5.10%), urine (4.46%) and body fluids (0.64%) (graph-1). Among *Non albicans Candida*, *Candida tropicalis* (52.86%) was most common *Candida* species to be isolated as biofilm producer followed by *C. Parapsilosis* (10.19%), *C. glabrata* (10.19%) and *C. krusei* (4.45%) while *C. albicans* was 35/157 (22.29%).

When *Candida* isolates were tested for biofilm formation capacity, biofilm production was most commonly observed for isolates of *C. glabrata* 16/18 (88.9%) and *C. krusei* 7/8 (87.5%) followed by *C. tropicalis* 87/145 (57.2%), *C. parapsilosis* 16/33 (48.5%) and *C. albicans* 35/109 (32.1%) isolates.

Antifungal resistance was observed more among biofilm producers. Fluconazole and (96.18%) and Ketoconazole (77.71%) were most resistant antifungal drugs in biofilm



Graph-1: Correlation of Biofilm Formation with Clinical Samples

	Biofilm producers	Fluconazole		Ketoconazole		Itraconazole		Amphotericin B		Nystatin	
		No.	%	No.	%	No.	%	No.	%	No.	%
Candida albicans	35	31	88.57	28	80.00	1	2.86	1	2.86	7	20.00
Candida tropicalis	83	82	98.80	58	69.88	4	4.82	7	8.43	20	24.10
Candida parapsilosis	16	16	100.00	14	87.50	4	25.00	1	6.25	4	25.00
Candida	16	15	93.75	15	93.75	2	12.50	2	12.50	5	31.25
Candida Krusei	7	7	100.00	7	100.00	1	14.29	1	14.29	2	28.57
Total	157	151	96.18	122	77.71	12	7.64	12	7.64	38	24.20

Table-1: Correlation of Biofilm Producer and Antifungal Resistance

producers. Itraconazole (7.64%), Amphotericin-B (7.64%) and Nystatin (31.93%) were found to be quite sensitive antifungal drugs in biofilm producers.

DISCUSSION

In this study, on the basis of Corn Meal Agar morphology and CHROMagar method, maximum number of isolates were identified as *C. tropicalis* 145 (46.33%) followed by *C. albicans* 109 (34.82%), *C. parapsilosis* 33(10.54%), *C. glabrata* 18(5.75%) and *C. krusei* 8(2.56%). On the basis of results obtained by different diagnostic methods, our study showed that out of total 313 isolates, *non albicans candida* species 204 (65.18%) were predominant isolates while *Candida albicans* was (34.82%) In our study this association was found to be statistically significant ($P < 0.05$). Similar results were obtained in study done by Manchanda *et al.*⁵ and Vijaya D *et al.*⁶ This change in pattern has been partly attributed to increased immune suppression resulting in higher numbers of susceptible immunocompromised patients, hospitalization especially in the ICUs, placement of central venous catheters and prophylactic use of antifungal agents in critically ill patient With the widespread use of broad-spectrum antimicrobials, the selective pressure exerted by the frequent use of antifungals also encourages the proliferation of drug-resistant *Non albicans Candida* species.⁷

A biofilm is a community of micro-organisms and their extracellular polymers that are attached to a surface.⁸ The ability to form biofilms is associated with the pathogenicity and considered as an important virulence determinant during Candidiasis. Biofilms may help fungi in maintaining the role of commensal and pathogen by evading host immune mechanisms, resisting antifungal treatment and withstanding the competitive pressure from other organisms.

The TCP (Tissue Culture Plate Method) method was considered the gold standard for this study and compared with data from TM (Tube Method). In the TCP method, the number of isolates showing biofilm formation were 157/313 (50.16%) and non-biofilm producers were 156/313 (49.84%). Tube method detected 91/313 (29.07%) isolates as biofilm producers and 222/313 (70.93%) as non-biofilm producers So, Tissue Culture Plate method (50.16%) was found to be more sensitive method for biofilm detection than Tube method (29.07%) and this finding was found to be statistically significant ($P < 0.05$). These observations were entirely in agreement with observations reported by Hassan *et al.*, 2011⁹, Ruzicka *et al.* 2004¹⁰ and Mathur *et al.*, 2006.¹¹ When biofilm production was studied with respect to clinical samples, maximum biofilm production was obtained in blood samples 87/157 (55.41%) followed by respiratory secretion 21/157 (13.38%), Catheter tip 20/ 157 (12.74%), pus 13/157 (8.28%), vaginal 8/ 157 (5.10%), urine 7/157 (4.46%) and body fluids 1/157 (.64%) The association of *Candida* biofilm production with different clinical samples was found to be statistically significant ($P < 0.05$). Similar results were obtained by Mohamed and Al-Ahmadey, 2013¹², Golia *et al.*, 2011¹³ and Singhai *et al.*, 2012.¹⁴ *Candida*

species are frequently found in the normal microbial flora of humans, which facilitates their encounter through implanted biomaterials and host surfaces.¹⁵ The devices become colonized by *Candida* which forms biofilm, the detachment of which can result in candidemia. Indwelling catheters therefore, represent a major risk factor associated with nosocomial *Candida* infections.

Biofilm formation was found to occur most frequently among *Non albicans Candida* species 122/157 (77.71%) than *C. albicans* 35/157(22.29%). Among *Non albicans Candida*, *Candida tropicalis* 83/ 157 (52.86%) was most common *Candida* species to be isolated as biofilm producer followed by *C. Parapsilosis* (10.19%), *C. glabrata* (10.19%) and *C. krusei* (4.45%). Similar results were obtained by Shin *et al.*, 2002¹⁶, Tumbarello *et al.*, 2007¹⁷ and Kumar and Menon, 2006.¹⁸

When fungal isolates were tested for biofilm formation capacity, biofilm production was most commonly observed for isolates of *C. glabrata* 16/ 18 (88.9%) and *C. krusei* 7/8 (87.5%) followed by *C. tropicalis* 87/145 (57.2%), *C. parapsilosis* 16/33 (48.5%) and *C. albicans* 35/109 (32.1%) isolates In our study, this association of biofilm formation capacity with different *Candida* species was found to be statistically significant ($P < 0.05$). Similar results were obtained by Tumbarello *et al.*, 2007¹⁷ and Kumar and Menon, 2006.¹⁸

The susceptibility of *Candida* strains to antifungal drug was performed by disc diffusion method as per CLSI M44-A2 protocol.²

In the present study, Antifungal resistance among biofilm producers and non-biofilm producers was compared. Fluconazole (151/157; 96.18%) and Ketoconazole (122/157; 77.71%) were most common antifungal drugs to be resistant among biofilm producers. Itraconazole (12/157; 7.64%), Amphotericin-B (12/157; 7.64%) and Nystatin (38/157; 31.93%) were found to be less resistant antifungal drugs among biofilm producers (table-1). Among non-biofilm producers, Maximum resistance was obtained for Fluconazole (11.54%) followed by Ketoconazole (0.64 %). No resistance was found for Itraconazole, Amphotericin B and Nystatin among non-biofilm producers. Similar results were obtained in study done by Kuhn *et al.*, 2002¹⁹ and Tumbarello *et al.*, 2007.¹⁷ So, biofilm production was found to be an important factor associated with antifungal resistance. Microorganisms organized in biofilms could become resistant to antifungals due to metabolic changes, reduction of their cell growth rate, expression of resistance genes and the presence of an extracellular matrix. Consequently, biofilm related infections are difficult to treat.

CONCLUSION

Biofilm production is an important risk factor for antifungal resistance. So, it is necessary to evaluate candida isolates for biofilm production. It will guide clinician for correct antifungal selection with exact doses that required. It will help in decrease the cost of treatment and proper management of patient.

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