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Detection of biofilm producing Candida species among different clinical isolates in Kidwai Memorial Institute of Oncology - A tertiary cancer care hospital

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Abstract

Background: The biofilm of organisms is considered as a virulence factor because of its resistance towards antimicrobial agents. Early detection of biofilm production is useful for clinical decision, as it is suggestive of potential pathogenic capacity of *Candida* isolates. **Aim:** 1. To detect the prevalence of biofilm producing *Candida* species isolated from clinical samples in cancer patients. 2. To compare two different methods, viz. tissue culture plate (TCP) method and tube method (TM) for biofilm detection. **Materials And Methods:** A total of 95 isolates of *Candida* species were recovered from diverse clinical sources which were analysed for biofilm formation by two standard methods, that is tissue culture plate method and tube method. **Results:** Biofilm was detected in isolates by tissue culture plate method (27%) and tube method (13%). Chi-square test was used and a P value of < 0.05 is considered as statistically significant. The estimated p value is 0.02, which is statistically significant indicating that the tissue culture plate method serves as a reliable quantitative tool for determining biofilm formation by clinical isolates of *Candida* species. **Conclusion:** As there is an increase in the number of patients who are immunocompromised receiving aggressive cancer chemotherapy, Candidiasis has emerged as an alarming opportunistic infection. From the two methods, tissue culture plate method is reliable for determining biofilm formation by *Candida* species in cancer patients which help in patient treatment modalities.

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Introduction

Candida albicans is the most common and prevalent species of the human microbiota; it asymptomatically colonizes the gastrointestinal tract, genitourinary tracts, oral cavity, and skin of most humans. Any alterations in host immunity, microbiota, stress and other factors can lead to overgrowth of *C. albicans* species, resulting in a wide range of infections, from superficial mucosal candidiasis to disseminated candidiasis.[1]

Immunosuppressed patients with solid organ and hematological malignancy, transplant recipients, are more susceptible for invasive candidiasis. Other vulnerable population for invasive candidiasis includes patients who had recent abdominal surgery, hemodialysis, people with a central venous catheter and parenteral nutrition.[2]

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Many nosocomial infections are associated with biofilms formation and attachment to medical devices and host tissues.[3]

Biofilms are the group of irreversible adherent cells with different structural and phenotypic properties as compared to free-floating planktonic cells. It is the predominant growth state of many microorganisms.[1]

Candida species are known to produce well-structured biofilms consisting of multiple types of cells and microbial species. It leads to an intrinsic resistance against various antifungal drugs and host immune defense.[4] It also inhibits the host immune system.[5]

There are various methods available to detect biofilm formation like tissue culture plate method, tube method, Congo Red Agar method, modified congo red agar method, bioluminescent assay, piezoelectric sensors, and fluorescent microscopic examination.[6]

However, it is not convenient to use all these methods in routine clinical laboratories. In this study, biofilm formation in cancer patients from various samples are detected by tissue culture plate method and tube method.

Biofilm Formation and Development

Biofilms are the complex three-dimensional structures. It is a collection of single or mixed species of microbial cells adherent to host tissues or abiotic surfaces (medical devices). Biofilms are embedded in an extracellular polysaccharide substance, thereby gives protection to the micro-organisms.[7]

Research in the field of biofilm formation by microorganisms has gained increasing momentum in recent decades. Traditionally, micro-organisms have been studied in free-floating (planktonic) cultures or as colonies grown on the surfaces of nutrient agar culture media, but it is now accepted that biofilms are the preferred growth state and it is the natural one for most micro-organisms.[8]

Formation of biofilms makes treatment difficult and causes increased rates of morbidity and mortality, thus representing one of the main virulence factors that contribute to the pathogenesis of candidiasis.[9]

Formation of biofilm by *Candida albicans* is a multifactorial process and it involves four major stages.[10]

1. Attachment of *Candida albicans* yeast cells to the surface.
2. Proliferation and filamentation of yeast cells.
3. Biofilm maturation and extracellular matrix formation.
4. Biofilm dispersion

The initial adherence of *Candida albicans* to a suitable surface is by means of several non-specific factors including electrostatic interactions, attractive and repulsive forces such as hydrophobic interactions, Brownian movement forces, and van der waals forces.[11]

Then the cell wall-associated adhesion molecules are expressed to strengthen the candida cell adhesion. The three important adhesion families involved are the hyphal wall protein (Hwp) family, the agglutinin-like sequence (Als) family, and the individual protein file family F/ hyphally regulated (Iff/Hyr) family.[12]

Following adhesion with the suitable surface, germination of yeast cell to hyphal and pseudo hyphal elements takes place. Als1p and Als3p plays a critical role in hyphal adhesion during biofilm formation.[13]

Biofilm formation is accompanied by changes in cellular morphology, cell number, and the production of extracellular matrix. The hyphal elements gives the structural integrity of the biofilm.[3]

Defective production of hyphal elements by mutation results in defective biofilm formation.[14]

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Extracellular matrix is necessary for the production of biofilm development and maturation. The components of extracellular matrix are proteins, carbohydrates, lipids, and nucleic acids secreted by cells within biofilm.[15]

Mannans are the most common polysaccharide present in extracellular matrix of *C.albicans* biofilms.[16]

Extracellular deoxyribonucleic acid produced by *C.albicans* enhances the stability of mature biofilm. It suggests that targeting extracellular deoxyribonucleic in the extracellular matrix may present a novel therapy to improve the activity of antifungal drugs.[17]

Finally, yeast cells are released from the mature biofilm and causes secondary and disseminated infections. These dispersed cells have increased potential to form biofilms, and damage endothelial cells in vitro. It also has reduced antifungal susceptibility.[18]

Biofilms has the potential to cause antifungal resistance. The known mechanisms of antifungal resistance by biofilm producing *Candida* species are increased efflux pump activity in fungus, mutations in genes encoding drug target enzymes, cell membrane and the cell wall composition alterations.[19]

Hawser and Douglas in 1955, were the first to demonstrate *Candida* biofilm resistance phenomenon in *C. albicans*.[20]

After this, the ability of biofilm forming *Candida* species to survive in high antifungal concentrations has been the subject for many researchers.[21,22] In the last decade, additional investigations began to focus on the role of biofilm-specific traits. Such as influence of growth rate reduction, high cell density, matrix extracellular production, nutrient limitation and gene expression alterations.[23]

Aim

1. To detect the prevalence of biofilm producing *Candida* species isolated from clinical samples in cancer patients.
2. To compare two different methods, viz. tissue culture plate (TCP) method and tube method (TM) for biofilm detection.

Materials And Methods

It is a cross sectional study conducted in a tertiary care cancer hospital for the period of 6 months (January 2021 – November 2021). Patient demographic details such as age, sex, and clinical information were collected.

Inclusion criteria: Cancer patients belonging to all age group.

Exclusion criteria: patients without cancer.

A total of 95 isolates of *Candida* species, recovered from diverse clinical samples (pus, throat swab, oral swab, urine, stool) is taken for the study. which are received for routine investigations in Microbiology department, Kidwai Memorial Institute of Oncology. Isolates from all samples which are morphologically resembling *Candida* will be identified by Gram's stain. The plates will be incubated at 37°C and 22°C for 24 hours. Culture with candidial growth will be subjected to Germ tube test to identify it as *albicans* and non-*albicans*. The isolated *Candida* species are stored in SDA deeps for furthur investigations. which were analysed for biofilm formation by two standard methods, that is tissue culture plate method and tube method.[6]

Detection of biofilm formation:

Tissue culture plate method:

Isolates from fresh agar plates were inoculated in brain heart infusion broth (BHIB) with 2% sucrose. It was the incubated at 37°C for 18–24 hours in a stationary condition. The broth with visible turbidity was diluted to 1 in 100 with fresh medium. Individual wells of flat bottom polystyrene plates were filled with 0.2 ml of the diluted cultures, and only broth

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served as a control to check sterility and nonspecific binding of the medium. These plates were incubated at 37°C for 24 hours. After incubation, the content of the well was removed and were washed 4 times with 0.2 ml of phosphate buffer saline (PBS pH 7.2) to remove free-floating "planktonic" bacteria. Biofilms formed by adherent "sessile" organisms in plate were fixed with 2% sodium acetate for half an hour and stained with 0.1% w/v crystal violet for another half hour. Excess stain was rinsed off by washing with deionized water and plates were kept for drying. Adherent bacterial cells usually formed a biofilm on all side wells and were uniformly stained with crystal violet.[6]

Optical densities (OD) of stained adherent bacteria were determined with a micro Enzyme-Linked Immunosorbent Assay auto reader at wavelength of 570 nm (OD 570 nm) and were graded as per Christensen *et al*.[24]

MEAN OPTICAL DENSITIES VALUE	BIOFILM FORMATION
<O.120	NONE / WEAK
0.120 – 0.240	MODERATE
≥0.240	HIGH

Tube method

In this method *Candida* isolates are inoculated in 10 ml of Brain Heart Infusion Broth (BHIB) with 2% sucrose and incubated at 37°C for 18 – 25 hours. The tubes are then decanted and washed with phosphate buffer saline (PBS pH 7.2). tubes are dried and Stained with crystal violet (0.1% w/v) for half an hour. Excess stain was removed; tubes were then dried. Biofilm formation was considered positive when a visible film lined the wall and bottom of the tube. Tubes were examined, and the amount of biofilm formation was scored as absent, moderate or strong.[6]

Germ tube test

A very light suspension of yeast like organism is made in 0.5-1.0 ml of sterile serum. It is then incubated at 35 - 37◦C for no longer than 3 hours. Place one drop of yeast serum mixture on a slide with a coverslip. Examine microscopically for germ tube production.

Germ tubes are the beginnings of true hyphae and appear as filaments that are not constricted at their points of origin on the parent cell.[25]

Statistical Analysis

Data was entered in Microsoft Excel 2019 and was analyzed using SPSS statistical software version 20.0.14. Quantitative variables were expressed as mean, median, range and standard deviation. Categorical variables were expressed as frequency and percentages. The association between the variables were assessed by using Chi-square test. The p value of <0.05 was considered as significant. The data were represented in the form of graphs and tables.

Results

In our study, a total of 95 *Candida* isolates were studied for biofilm formation. Age range was from 10-80 years with 45 females and 50 males. Male: Female ratio 1.1: 1. Most common age group infected with *Candida* is between 61 to 70 years.

Biofilm was detected in isolates by tissue culture plate method (27%) and tube method (13%). Chi-square test was used and a P value of < 0.05 considered as statistically significant. The estimated p value is 0.02, which is statistically significant indicating that the tissue culture plate method serves as a reliable quantitative tool for determining biofilm formation by clinical isolates of *Candida* species.

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ENT (oral and throat swabs) and sputum were the most common type of sample sent for culture and sensitivity. Most of the biofilm producers were isolated from ENT and sputum. By germ tube test, out of 95 *Candida* isolates, 39 (41%) were *C.albicans*, out of this, 11 were biofilm positive.

Table 1: Gender wise distribution of cases

GENDER	TOTAL SAMPLES (n = 95)		BIOFILM POSITIVE (n = 26)	
	N	%	N	%
MALE	50	52.64%	15	57.69%
FEMALE	45	47.36%	11	42.31%
TOTAL	95	100%	26	100%

Table 2: Age wise distribution of cases

AGE RANGE	TOTAL (n = 95)		BIOFILM POSITIVE (n = 26)	
	N	%	N	%
1-10	1	1.05%	0	0
11-20	3	3.16%	1	3.85%
21-30	4	4.21%	2	7.69%
31-40	8	8.42%	1	3.85%
41-50	19	20%	4	15.38%
51-60	21	22.11%	5	19.23%/
61-70	24	25.26%	9	34.62%
71-80	15	15.79%	4	15.38%
Total	95	100%	26	100%

Table 3: Sample type analyzed

SAMPLES	TOTAL SAMPLES (n = 95)		BIOFILM POSITIVE (n = 26)	
	N	%	N	%
ENT	47	49.47%	11	42.31%
SPUTUM	33	34.74%	10	38.46%
PUS	9	9.47%	2	7.69%
STOOL	6	6.32%	3	11.54%
TOTAL	95	100%	26	100%

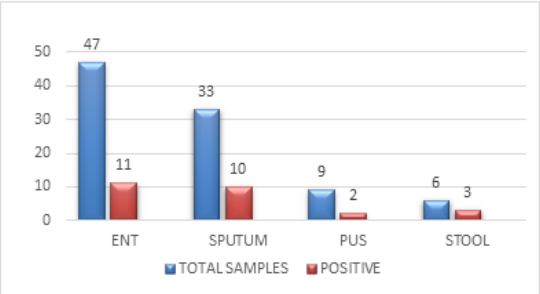

Figure 3: Sample type analyzed

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Table 4: Biofilm production by tissue culture plate method and tube adherence method

BIOFILM FORMATION	TISSUE CULTURE PLATE METHOD (n=26)		TUBE METHOD (n=13)	
	n	%	n	%
HIGH	2	2.1%	1	1%
MODERATE	24	25.3%	12	2.6%
WEAK/NONE	69	72.6%	82	86.3%
TOTAL POSITIVE	26	27%	13	13%

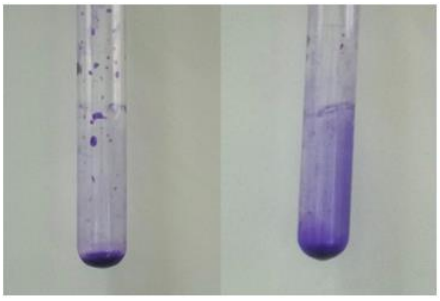

Figure 2: Tube adherence method: negative and positive results

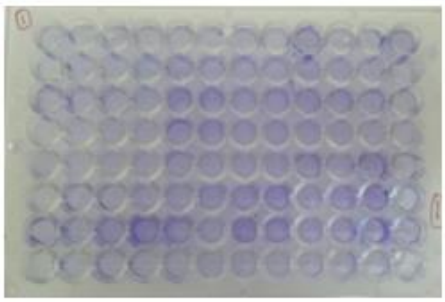

Figure 3: Microtiter plate assay indicating biofilm production

Table 5: Biofilm production in *Candida albicans* and *non albicans* species

Candida species	Tube method		Tissue culture plate method	
	Positive	Negative	Positive	Negative
Candida albicans	7	32	11	28
Non albicans *Candida*	6	50	15	41

Discussion

Invasive fungal infections are an emerging cause of morbidity and mortality in neutropenic patients with malignancies. Recently, production of biofilm by *Candida* species and inadequate antifungal therapy have been described as independent mortality factor in candidemia patients.[26]

In our study, we studied 95 samples exclusively and compared tube method and tissue culture plate method for biofilm detection, that can be used in routine clinical laboratories. The age group with the highest number of isolates was 61-70 years (25.26%). Males (52.64%) are more commonly infected than females (47.36%). Oral and throat swabs (49.47%) followed by sputum (34.74%) had the highest frequency of *Candida* isolates in the study. Biofilm was detected in isolates by tissue culture plate method (27%) and tube method (13%). Chi-square

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test was used and a P value of < 0.05 considered as statistically significant. The tissue culture plate method was found to be most sensitive than tube method.

In our study, out of 95 *Candida* isolates, 39 (41%) were *C.albicans*, it is similar to that of the study conducted by Munmun et al, among 90 *Candida* species isolated, most predominant species was found to be *C.albicans* (45.5%). In this study *Candida* spp. were isolated from urine (43%), BAL/sputum (18.88%), high vaginal swab (8.88%), suction tips (7.77%), blood and wound swabs (6.66%), pus (3.33%), bile aspirate (2.22%), and deep tissue (1.11%). A larger number of females were affected than males. These findings are contrast to our study.[27]

In the study conducted by Renuka devi et al, out of 64 *Candida* isolates, 47 (73.4%) isolates were biofilm producers.[26]

In the study conducted by Janakiram et al, out of 50 *Candida* isolates, 37 (74%) isolates were positive for biofilm. It is higher than our study.[28]

In the study conducted by Shilpa khatri, 49 (61.25%) out of 80 *Candida* isolates obtained from the clinical specimens produced biofilm.[29]

In the study conducted by Vinitha et al in which a total of 81(73%) out of 111 *Candida* species isolates obtained from the clinical isolates produced biofilm in non-cancerous patients.[30]

Conclusion

As there is an increase in the number of patients who are immunocompromised receiving aggressive cancer chemotherapy, Candidiasis has emerged as an alarming opportunistic infection. The ability of *C.albicans* to form biofilms further complicates treatment of these infections and contributes to the increased mortality rates. The presence of a biofilm matrix is the main defining feature of *C.albicans* biofilms. Early diagnosis and adequate anti-fungal treatment will improve patient outcome. Detecting biofilm production of *Candida* species helps us to plan treatment and identify the niche for production of biofilms. From the two methods of biofilm detection, tissue culture plate method is reliable for determining biofilm formation by *Candida* species in cancer patients.

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