DOI: 10.14260/jemds/2015/2065

ORIGINAL ARTICLE

ANALYSING THREE DIFFERENT SCREENING METHODS FOR BIOFILM FORMATION IN CLINICAL ISOLATES OF *CANDIDA*

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HOW TO CITE THIS ARTICLE:
Shilpa Khatri, Sumana M. N, Rashmi P. Mahale, Arnaw Kishore. "Analysing Three Different Screening Methods for Biofilm Formation in Clinical Isolates of *Candida*". Journal of Evolution of Medical and Dental Sciences 2015; Vol. 4, Issue 83, October 15; Page: 14515-14524, DOI: 10.14260/jemds/2015/2065

ABSTRACT: Biofilm formation helps the microbes to escape host defenses and develop resistance against antimicrobial agents. Early detection of slime production by the *Candida* species may direct the clinical management. The study was taken with the aim to estimate biofilm formation by *Candida* species in indwelling catheters and to compare three methods i.e. Congo red agar (CRA) method, Tissue culture plate (TCP) method, and tube method (TM). In this study, we evaluated the reliability of these methods in order to determine most suitable screening method and noted the sensitivity pattern of these isolates with the help of Vitek–2. Our data indicates that the TCP is an accurate and reproducible method for screening and can serve as a reliable quantitative tool for determining bio film formation by clinical isolates of *Candida* species along with susceptibility testing to reduce resistance pattern.

KEYWORDS: *Candida albicans*, Non *albicans Candida*, Tissue Culture Plate Method, Tube Method, Congo Red Agar Method, Evaluation.

INTRODUCTION: In order to survive and have its own progeny, almost all the pathogenic microbes have developed techniques to assist in colonization, invasion, and pathogenesis which aid as an effective virulence factors and *Candida* and its species is not uncommon. Pathogenic *Candida* spp. can cause infections ranging from superficial to deep-seated mycoses. The fourth most common recognized cause of nosocomial invasive infection is *Candida* species.[1] Over the last two decades there has been an increase in the incidence of *Candida* infection in different parts of the world and in various clinical settings.[2] Diabetes mellitus, immune-compromised states, antibiotic use, indwelling devices, intravenous drug use, and hyper-alimentation fluid are few risk factors which lead to *Candida* infection.

Although *C. albicans* is most often associated with serious fungal infection, in recent time other *Candida* species also have emerged as clinically important opportunistic pathogens.[3] The formation of surface-attached microbial communities known as "biofilm" is one of the important factors contributing to the virulence of *Candida* species.[4] These are made up of layers of cells, which are embedded within a matrix of extracellular polymeric material.

The ability to form extensive biofilm on catheters and other prosthetic devices, contribute its prevalence as an aetiological agent of intravascular nosocomial infection.[5] *Candida* produces large quantities of sticky, glutinous material in glucose containing solutions. *Candida* species are frequently found in the normal inhabitat of humans, which facilitates their encounter with most implanted biomaterials, host surfaces and with biofilm formation, this helps to evade host defences, exist as a persistent source of infection and develop resistance against antifungal agents.[6] Biofilm producing resistant *Candida* species represents a major challenge to health care personnel and pharmaceuticals company especially in the design of therapeutic and prophylactic strategies, failure of which result in high mortality as well as economic problem due to prolonged hospital stay.[7] This study was

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undertaken with the aim to estimate biofilm formation by *Candida* species in indwelling catheters.

MATERIALS AND METHODS: A descriptive study was done over a period of a year in a tertiary care hospital at department of Microbiology, after obtaining clearance from Institutional ethical committee. A total of 80 patients admitted in critical care units for more than 48 hours who were on Foley's catheter, endotracheal tubes, and intravenous catheter whose sample yielded *Candida* were included in the study. Detailed history of patients with age, sex, and days of catheterization was recorded. The *Candida* isolates obtained were further identified by conventional methods such as germ tube test, microscopic morphology on cornmeal agar and sugar fermentation and assimilation tests, phospholipase estimation and proteinase estimation. [8] Anti-fungal susceptibility was also done against fluconazole, flucytosine, voriconazole, amphotericin B and caspofungin in VITEK–2 compact systems. Following interpretive susceptible criteria for anti-fungal were used.

Organism	Sensitive	Intermediate	Resistant
Caspofungin Calling range 0.25 to 4µg/ml (Available in VITEK AST YS 06)			
C. albicans	≤0.25	0.5	≥1
C. glabrata	≤0.12	0.25	≥0.5
C. tropicalis	≤0.25	0.5	≥1
C. krusei	≤0.25	0.5	≥1
C. parapsilosis	≤2	4	≥8
C. guilliermondii	≤2	4	≥8
Fluconazole calling range 1 to 64 µg/ml			
C. albicans	≤2	4	≥8
C. glabrata	≤32	32	≥64
C. krusei	-	-	-
C. parapsilosis	≤2	4	≥8
C. tropicalis	≤2	4	≥8
Voriconazole CALLING RANGE 0.12 to 8 µg/ml			
C. albicans	≤0.12	0.25–0.5	≥1
C. glabratae	-	-	-
C. krusei	≤0.5	1	≥2
C. parapsilosis	≤0.12	0.25–0.5	≥1
C. tropicalis	≤0.12	0.25–0.5	≥1

Table 1: Break Point Update For Anti-Fungal Based on CLSI M27-S4[9]

For flucytosine, isolates showing MIC's ≤4µg/ml were considered as susceptible, 8-16µg/ml as intermediate and ≥32µg/ml as resistant. For amphotericin B, isolates showing a MIC of ≤1.0µg/ml were taken as susceptible and those with MIC>1µg/ml were considered as resistant.[10, 11] MIC interpretative criteria were referred to those described in the CLSI document M 27-S3 for amphotericin B, 5-flucytosine.[12]

All the cultures of *Candida* species were maintained on Sabouraud's dextrose agar (SDA) slopes for future references. Congo red agar method (CRA), Tissue culture plate method (TCP), and Tube method (TM) were done with *Candida* species from indwelling catheters to evaluate the reliability of biofilm formation.

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Tissue Culture Plate Method (TCP): Isolates from freshly sub cultured plates were inoculated in Sabouraud's Dextrose Broth (SDB) with 8% glucose and incubated for 24 hours at 37˚C in stationary conditions and then diluted to 1:100. Each well of sterile polystyrene 96 well flat bottom micro titre plates was filled with 200µl aliquots of diluted culture. Uninoculated media served as a negative control which was also inoculated in triplicate. The micro titre plate was incubated for 48 hrs at 37˚C. After washing the wells for 3 times with 200µl of phosphate buffer saline (PBS) at pH 7.2, the floating planktonic bacteria were removed. The biofilms thus formed in plates were fixed using 2% w/v sodium acetate for 10 minutes and stained with 0.1% w/v crystal violet for 10 minutes. After washing thoroughly with de- ionized water to remove any excess stain, the plates were dried. Micro-ELISA auto-reader at the wavelength of 540 nm was used to measure the optical density (OD) of the stained adherent micro-organisms. The mean value from a control well was deducted from all test OD 540 values. These values were considered as an index of bacteria adhering to surface and forming biofilms. Experiments were performed in triplicate.[12,13] The OD value were calculated as mentioned in Table 1.

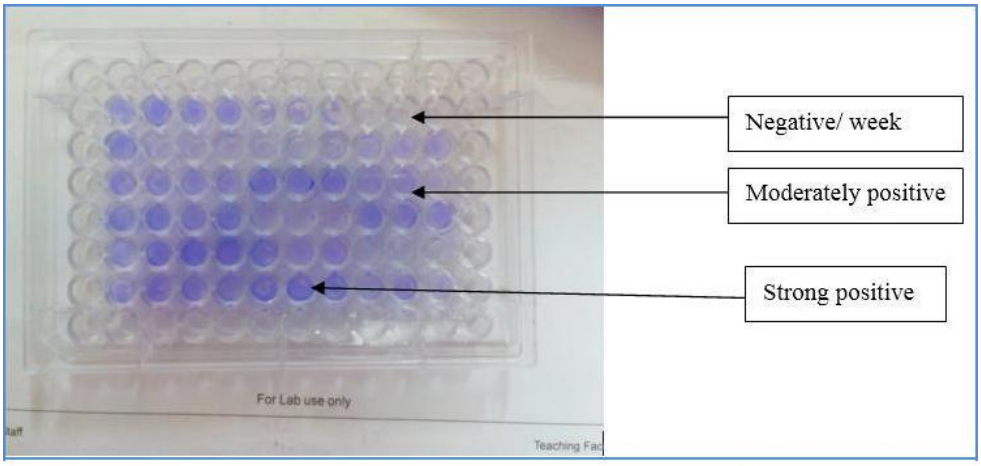

Fig. 1: Tissue Culture Plate Method

Mean OD	Adherence	Bioflim formation
<0.120	Non/ weak	Non/ weak
0.120- 0.0240	Moderate	Moderate
>0.240	Strong	High

Table 1: Interpretation of Biofilm Production[12]

Tube Method (TM): A quantitative assessment of biofilm formation was determined as described by Christensen et al[13] SDB with 8% glucose was inoculated with loopful of microorganisms from overnight culture plates incubated for 48 hrs at 37^0C. Tubes were decanted and washed with PBS and dried tubes were stained with 0.1% crystal violet. Excess stain was removed and tubes were washed with deionized water, tubes were then inverted dried and observed for biofilm formation. Biofilm formation was considered positive when visible film lined the wall and bottom of the tube. Formation of ring at the liquid interface was not indicative of biofilm formation. A tube containing only SDB without any inoculum was taken as negative control. Experiments were performed in triplicate.

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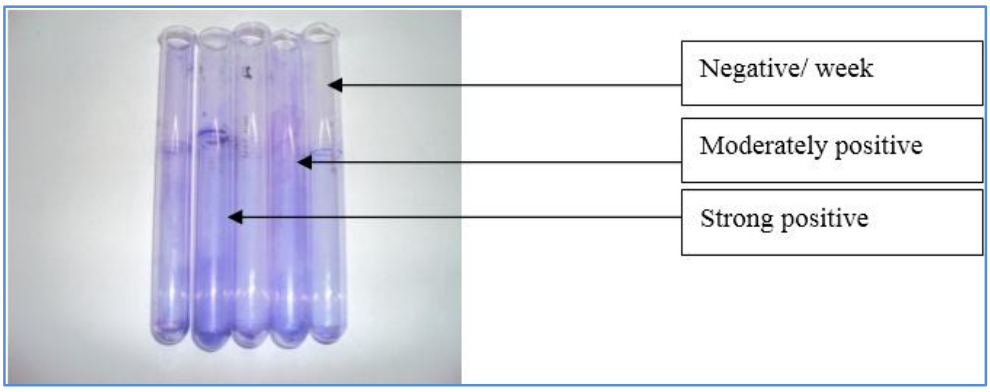

Fig. 2: Tube Method

Congo red Agar Method (CRA): Freeman et al[14] had described an alternative method of screening biofilm formation by *Candida* isolates. In the present study the Congo red agar (CRA) was optimized to get strong black pigmentation at 48hrs incubation and then for 2-4 days room temperature. Black coloured colonies with dry crystalline consistency interpreted as positive biofilm producing strains. Red coloured colonies- interpreted as negative for biofilm production**.**

Fig. 3

RESULTS: Among the 80 *Candida* isolates, 58 (72.5%) were non- *albicans Candida* species and 22 (27.5%) were *Candida albicans*. Among the non- *albicans Candida* species, the most common isolate was *C. tropicalis* 38 (47.5%) followed by *C. famata* 09 (11.25%). Other species isolated were *C. parapsilosis* 04 (5%), *C. glabrata* 03 (3.75%), *C. krusei* 02 (2.5%) and *C. lusitaneae* 02 (2.5%) as in Graph 1 A. Out of 80 *Candida* species tested Biofilm production was found to occur most frequently among non-*albicans Candida*, 38 (47.5%) than *Candida albicans* 11 (13.75%). Among the non-*albicans Candida* species, *C. tropicalis* 27 (33.75%) was the highest biofilm producer Graph 1 B.

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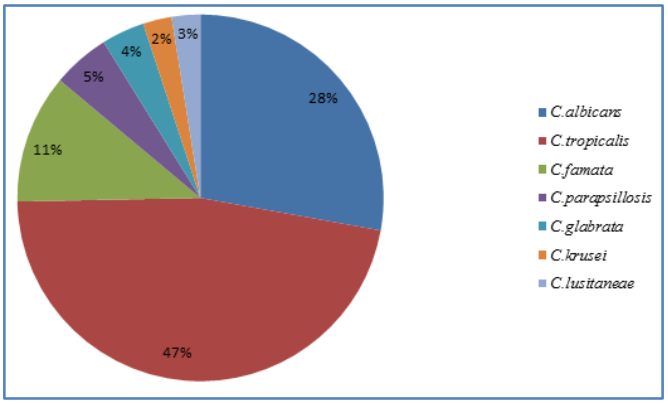
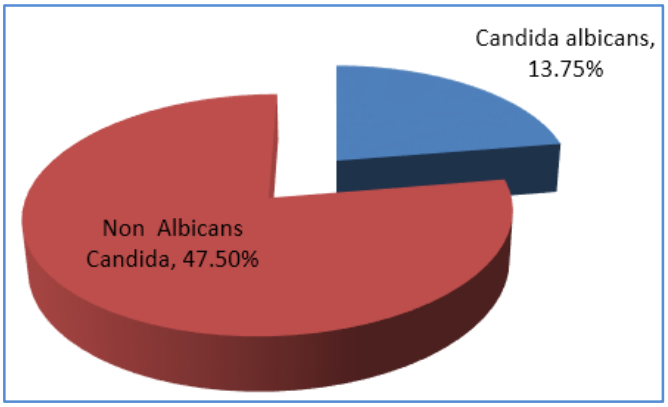
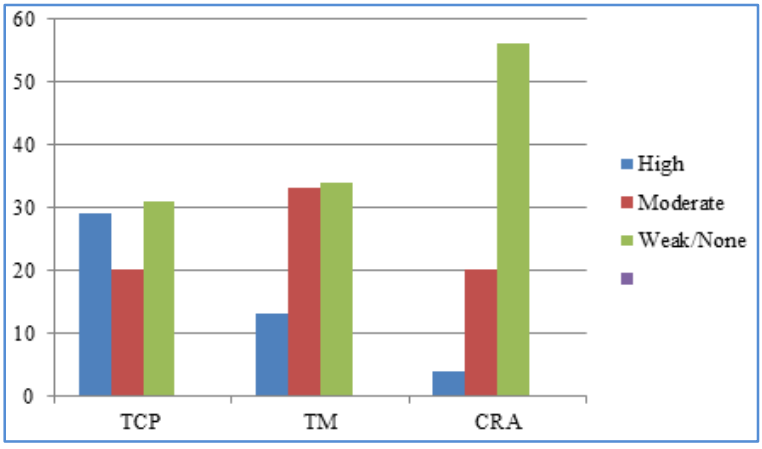
Graph 1 A Various *Candida* species



Graph 1 B: Biofilm Production by *Candida albicans* and Non-*Candida albicans* Species



Graph 2: Screening of the isolates for biofilm formation by Tissue culture plate method, Tube method and Congo red Agar methods.



Graph 2

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Tissue Culture Plate Method, the standard method detected 29 as strong and 20 as moderate biofilm producers. By TM, the number of strong biofilm producers were 13, moderate were 33 and weak or non-biofilm producers were 34. Very different results were observed by the CRA method, with which only 04 isolates showed black colonies with crystalline appearance**.** Figure 3.

Screening of the isolates for biofilm formation by Tissue Culture Plate, Tube Method and Congo Red Agar methods is as mentioned in Table 2A & Graph 2. In the standard TCP assay only 49 (61.25%) of total tested isolate displayed positive biofilm, which is very close to TM where 46 (57.50%) isolates showed positive result, showing a strong co-relation. In case of CRA method only 28(35%) isolates showed positive biofilm. The species level screening of biofilm formation is as shown in Table 2B. We observed that there are 11 biofilm producing C.*albicans* and 38 Non *albicans Candida* as in Table 2C.

	Biofilm Formation	**TCP (%)**	**TM (%)**	**CRA (%)**
No. of Isolates	HIGH	29	13	4
	MODERATE	20	33	20
	WEAK/NONE	31	34	56

Table 2A: Screening of the isolates for biofilm formation by Tissue Culture Plate, Tube Method and Congo Red Agar methods

Method / Species	**Strong**			**Moderate**			**Weak/negative**		
	TCP	**TM**	**CRA**	**TCP**	**TM**	**CRA**	**TCP**	**TM**	**CRA**
C.albicans	07	04	02	04	07	00	11	11	20
C. tropicalis	15	06	02	12	19	05	11	13	31
C.famata	04	03	00	02	02	09	03	04	00
C.parapsillosis	01	00	00	00	01	03	03	03	01
C.glabrata.	00	00	00	01	01	01	02	02	02
C.krusei.	01	00	00	01	02	00	00	00	02
C.lusitaneae	01	00	00	00	01	02	01	01	00
Total	**29**	**13**	**4**	**20**	**33**	**20**	**31**	**34**	**56**

Table 2B: Species level screening of the isolates for biofilm formation by Tissue culture plate method, Tube method and Congo red agar methods

TCP – Tissue Culture Plate Method TM – Tube method CRA – Congo Red Agar Method

Method / Species	**POSITIVE**	
	TCP	**TM**
C.*albicans*	11	11
Non *albicans Candida*	38	35

Table 2C: *Candida* vs Non *albicans Candida* Biofilm producer

On observation the resistance pattern among biofilm producing C. *albicans*, more than 60% and 70% are resistance to Fluconazole and Flucytosine while almost 20% are resistance to Voriconazole and Caspofungin. The resistance for the same antifungal was seen more in *C. tropicalis*

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where almost 75%, 80% and 30% resistance was seen to Fluconazole, Flucytosine and Voriconazole but in case of Caspofungin the resistance was quite low. On further analysis we observed that *Candida* spp has become resistance to Fluconazole, once a drug of choice. We were not able to record the sensitivity pattern of *C*. *famata* with Vitek 2 (Biomerieux, Marcy I Etoile, France). The antifungal susceptibility results showed highest resistance to Fluconazole, Flucytosine and Voriconazole although Caspofungin, Micafungin and Amp-B showed good efficacy.[10] Tables describing resistance pattern among biofilm producer and non bioilm producer is as given in Table 3A and 3B.

Antifungal / Species Name	Total isolates	Fluconazole n (%)	Flucytosine n (%)	Variconazole n (%)	Caspofungin n (%)	Amphoterecin Bn (%)	Micafungin n (%)
C.albicans	11	07 (63.63)	08 (72.72)	02 (18.18)	02 (18.18)	01 (9.09)	02 (18.18)
C. tropicalis	27	20 (74.07)	21 (77.77)	08 (29.62)	01 (3.70)	03 (11.11)	01 (3.70)
C.famata	00	00 (00)	00 (00)	00 (00)	00 (00)	00 (00)	00 (00)
C.parapsillosis	03	01 (33.33)	00 (00)	00 (00)	00 (00)	00 (00)	00 (00)
C.glabrata.	02	01 (50)	01 (50)	00 (00)	00 (00)	00 (00)	00 (00)
C.krusei.	02	02 (100)	00 (00)	00 (00)	00 (00)	01 (50)	00 (00)
C.lusitaneae	01	00 (00)	00 (00)	00 (00)	01 (50)	00 (00)	01 (50)

Table 3A: Resistance pattern (%) of biofilm producing *Candida*

Antifungal / Species Name	Total isolates	Fluconazole n (%)	Flucytosine n (%)	Variconazole n (%)	Caspofungin n (%)	Amphoterecin Bn (%)	Micafungin n (%)
C.albicans	11	2 (18)	3 (27)	00 (00)	00 (00)	00 (00)	00 (00)
C. tropicalis	11	4 (36)	3 (27)	01 (09)	01 (09)	01 (09)	01 (09)
C.famata	03	00 (00)	00 (00)	00 (00)	00 (00)	00 (00)	00 (00)
C.parapsillosis	01	1 (100)	1 (100)	00 (00)	00 (00)	00 (00)	00 (00)
C.glabrata.	01	1 (100)	00 (00)	00 (00)	00 (00)	00 (00)	00 (00)
C.krusei.	00	00 (00)	00 (00)	00 (00)	00 (00)	00 (00)	00 (00)
C.lusitaneae	01	00 (00)	01	00 (00)	00 (00)	00 (00)	00 (00)

Table 3B: Resistance pattern (%) of non-biofilm producing *Candida*

DISCUSSION: *Candida* and its species are asexual, diploid, dimorphic fungus which is very commonly present in humans and their environment. A relatively small number of *Candida* species are pathogenic for humans which are responsible for causing a variety of superficial and deep-seated mycoses[15]. *Candida* organisms are usually commensals; but to act as pathogens, interruption of normal host defences is necessary.

With the increase in number of patients who are immunocompromised, aged, receiving prolonged antibacterial and aggressive cancer chemotherapy or undergoing invasive surgical procedures and organ transplantation *Candida* infection has emerged as an alarming opportunistic disease particularly in these patients.[16] Biofilm producing microorganisms are responsible for many

recalcitrant infections and are notoriously difficult to eradicate. They exhibit resistance to antibiotics and antifungals by various methods like restricted penetration of drugs, decreased growth rate and expression of resistance genes. A biofilm is a community of microorganisms and their extra cellular polymers that are attached to a surface.[17] In our study the resistance pattern of *Candida* has shown highest resistance to Fluconazole, Flucytosine and Voriconazole although Caspofungin and Amp-B showed good efficacy. An Indian study by Adhikary R et al had reported very high resistance to Voriconazole (56%) and Fluconazole (36%).[18] We observed higher degree of resistance to Fluconazole in biofilm producers as compared to non-producers as reported by several authors.[19]

If we compare and corelate the TCP method then we found that our study is correlated with Vinitha et al[20] in which a total of 81(73%) out of 111 *Candida* species isolates obtained from the clinical isolates produced biofilm. We have got 49(61.25%) out of 80 *Candida* species isolates obtained from the clinical isolates produced biofilm with high resistance pattern among biofilm producers. In a paper on Staphylococcus aureus by M Gogoi et al The TCP, TM and CRA detected 61.7%, 41.7% and 18.2% of biofilm producers, respectively which co relates with our study.[20] Newer techniques like DNA extraction and quantification, qPCR etc. evaluated of late by other authors, which is time consuming and expensive.[21,22]

A study by Mathur et al[23] the TCP assay, only 7(4.6 %) of 152 tested *Staphylococcus* spp isolates displayed a biofilm positive phenotype, in contrast to our study where 49(61.25%) isolates were biofilm positive. The TM correlates well with the TCP test for strongly biofilm producing isolates but it was difficult to discriminate between weak and biofilm negative isolates due to the variability in observed results by different observers. In accordance with the preceding studies, TM and CRA cannot be suggested as general screening test to identify biofilm producing isolates.

CONCLUSION: *Candida* biofilms are seen in most of the medical devices and indwelling catheters. We compared the three methods employed in our study viz TCP, TM and CRA. On comparing; the TCP method emerged as most sensitive, most reproducible, accurate, efficient and specific method to detect the biofilm production and can be recommended as a general screening method for detection of biofilm producing *Candida* in laboratories.

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FINANCIAL OR OTHER COMPETING INTERESTS: None

Date of Submission: 07/08/2015.
Date of Peer Review: 08/08/2015.
Date of Acceptance: 05/10/2015.
Date of Publishing: 14/10/2015.