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Original Research Paper

Microbiology

STUDY OF BIOFILM PRODUCING PROPERTY OF CANDIDA SPECIES ISOLATED FROM URINE SAMPLES OF CATHETERIZED PATIENTS

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ABSTRACT In this study, clinical isolates of *Candida* were collected from the urine samples of catheterized patients. The *Candida* isolates obtained were further identified by conventional methods such as gram stain (direct microscopy), germ tube test, Sabouraud dextrose broth. The study demonstrates that biofilm formation and consequent infections of medical indwelling devices is a serious problem in hospitals in this era.

KEYWORDS : Urinary Tract Infections, Bioflim, Candida

INTRODUCTION

Urinary Tract Infection (UTI) is the most common type of nosocomial infection [1]. 10 to 15% of UTIs are caused by *Candida* species.[2] Candiduria is seldom encountered in healthy individuals. Candida organisms are commensals; and to act as pathogens, interruption of normal host defences is necessary. Therefore, risk factors for Candida infections include immune-compromised states, diabetes mellitus, and iatrogenic factors like antibiotic use, indwelling devices, intravenous drug use, and hyper alimentation fluids [3]. Candidal lower urinary tract infection (UTI) is quite frequently seen in association with indwelling catheters, and may originate from the gastrointestinal or genital biota. The frequency of urinary tract infections (UTIs) due to Candida species is increasing and these infections are now being the most common clinical finding, particularly in hospitalized patients.[4]

Although the majority of implant infections are caused by gram-positive bacteria, mainly staphylococci, infections due to Gram-negative bacteria and fungi tend to be more serious. Fungal infections are most commonly caused by the pathogenic Candida species, particularly C. albicans, C. tropicalis and C. parapsilosis. These organisms are regarded as increasingly important nosocomial pathogens. [5]

Urinary catheters are tubular latex or silicone devices, which when inserted may readily acquire biofilms on the inner or outer surfaces. The longer the urinary catheter remains in place, the greater is the tendency of these organisms to form biofilms and cause urinary tract infections. [6]

Biofilms are microbial derived sessile communities characterised by the cells that are irreversibly attached to a substratum or to each other embedded in a matrix of extracellular polymeric substance produced by them and exhibit an altered phenotype with respect to growth rate and gene transcription [7] Biofilm may form on any implantable device and majority of nosocomial infections are associated with biofilm infections of medical devices [8].By producing a biofilm, the micro-organism creates a shield to protect itself.[9] Formation of a biofilm is a virulence factor of a microorganism. Biofilm is produced by both fungi and bacteria .In mycology, Candida spp. forms most common fungal biofilm which is extremely difficult to treat[10]. Biofilms of *Candida* species exhibits resistance to antifungal agents [11].

Biofilm formation on medical devices can negatively impact the host not only by causing the failure of the device but also by serving as a reservoir or source for future continuing infections [12]

Formation of Biofilm exhibits increased resistance to commonly available antifungal therapies; these infections are very hard to treat. Most often, treatment of biofilm infections involves the removal of the infected medical devices [13]. The majority of medically important Candida spp. has now been shown to develop biofilm including *C. albicans and* Non albicans Candida which includes *C. krusei*, *C. glabrata*, *C. tropicalis C. dubliniensis and C. parapsilosis* [14].

The detection of biofilm becomes very necessary for the treatment of infection [15]. Various Phenotypic methods are used for the routine detection of Biofilms are - Microtiter Plate Method, Congo Red Agar and Tube adherence method.

The present study was conducted with the aim of identification of *Candida* species isolated from urine samples of catheterised patients, comparison of three phenotypic methods for biofilm detection in these *Candida* isolates.

MATERIAL AND METHOD

The present study was conducted over a period of 6 months (January 2018 to june 2018) in the Department of Microbiology, Sawai man singh Medical College & Hospital, Jaipur, Rajasthan. In this study, a total of 50 clinical isolates of *Candida* were collected from the urine samples of catheterized patients. The *Candida* isolates obtained were further identified by conventional methods such as gram stain (direct microscopy), germ tube test, Sabouraud dextrose broth. In microscopy budding yeast like cells are seen with the help of gram's stain, and then inoculated on Sabourad's Dextrose Agar with Chloramphenicol at 37˚C for 24 hours. For initial speciation, Germ tube test was done. C.albicans produce germ tubes from their yeast cells when placed in a liquid enviornment and incubated at 37ºC for 1-2 hours. It is also known as Reynolds Braude phenomenon. Candida species when inoculated into SDB (Subourauds dextrose broth) medium that contains high glucose (8%) and protein (1%); adheres to the polystyrene tubes and plates and form biofilms.

Hichrome agar- Simultaneously Candida Spp. were inoculated on Candida CHROM agar and incubated at 37˚C for 24 hrs and the species were identified by colour of the colonies on CHROM agar media as per manufacturer's instructions (TM Media).

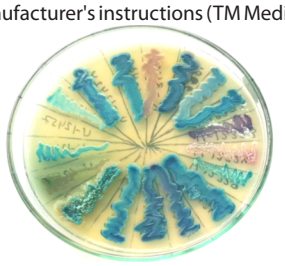


Fig.1 Hichrome plate for candida speciation

Biofilm Formation- Biofilm formation was detected by three methods as described below

Tissue culture plate method

Isolates from freshly sub-cultured plates were inoculated in Subarouds dextrose broth (SDB) with 8% w/v glucose and incubated for 24 hours at 37˚C in stationary conditions and then diluted to 1:100 with fresh SDB. Individual wells of sterile polystyrene 96 well flat bottom microtitre plates were filled with 200µl aliquots of diluted culture. Un-inoculated SDB served as a control to check sterility and nonspecific binding of media. Control strains were also inoculated in triplicate.

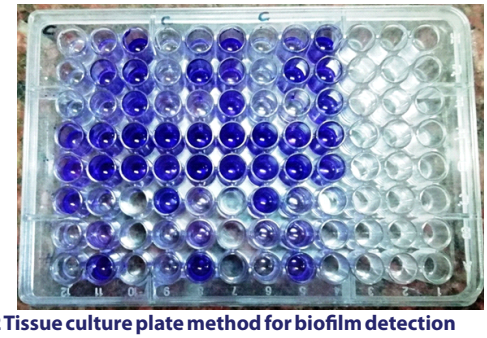


Fig.2 Tissue culture plate method for biofilm detection

The microtitre plate was incubated for 24 hours at 37˚C. After incubation contents of each well was removed by tapping the plates. After washing the wells for three times with 200µl of phosphate buffer saline (PBS pH 7.2), the floating planktonic bacteria were removed.The biofilms thus formed in plates were fixed using cold methanol for 10 minutes and then tainted with 0.1% w/v crystal violet for 10 minutes at room temprature. After washing thoroughly with de- ionized water to remove any excess stain, the plates were dried and 250 µl of 95% ethanol was added to each stained well. Dye was allowed to solubilise by covering plates and incubating for 15 min at room temperature. Based on the optical density (OD)produced by bacterial films at a wavelength of 620 nm, isolates were classified into the following categories .Non –BF producers ,weak, moderate or high biofilm producers as described by Stepanovic et al. 2000[16]. Experiments were performed in triplicate.

Congo Red Agar Method (CRA): Freeman et al.[17] had described an alternative method of screening biofilm formation by *Candida* isolates, which requires the use of a specially prepared solid medium—brain–heart infusion (BHI) broth supplemented with 8% glucose and Congo red. The medium was composed of BHI (37 g/L),glucose (80 g/L), agar no.1 (10 g/L), and Congo red stain(0.8 g/L). Congo red was prepared as concentrated aqueoussolution and autoclaved at 121°C for 15 +min separately from other medium constituents and then added when the agar had cooled to 55°C. Plates were inoculated and incubated aerobically for 48 h at 37°C. Black coloured colonies with dry crystalline consistency interpreted as positive biofilm producing strains and negative results were indicated by white or very light pink-colored colonies.

Tube adherence method

A Qualitative assessment of Biofilm production was detected by tube method described by Christensen et al. loopful of organisms from SDA were inoculated into Sabouraud's Dextrose broth supplemented with glucose (final concentration 8%). The tubes were incubated at 370C for 48 hours after which the glass tubes were decanted and stained by 0.1%crystal violet for half an hour washed with distilled water three times and dried. Tubes are examined for biofilm production. Biofilm production is tested twice and read independently by two different observers. A positive result was defined as the presence of a layer of stained material adhered to the inner wall of tubes. The adherent biofilm layer is scored visually as negative or positive (1+), moderate positive (2+) or strong positive (3+) [11].

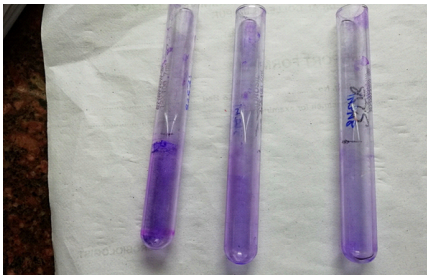


Fig. 4 Tube method for biofilm detection

The TCP method was considered the gold-standard for this study and compared with data from TM and CRA methods.(Parameters like sensitivity, specificity, negative predictive value, positive predictive value were calculated).

RESULTS

Out of 50 *Candida species*, 18(36%) were *Candida albicans* & 32(64%) were *non-albicans Candida*.

Germ tube test: 18 (32%) out of 50 showed production of germ tube. All the GTT positive *Candida* isolates were identified as *Candida albicans* by HiChrom agar.

	number	Percentage%
Candida albicans	18	32
Non albicans candida	32	64
Total	50	100

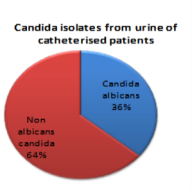


Table /Graph 1. Candida isolates from urine of catheterized patients

Identification by HiChrom Agar: Non-albicans *Candida* (NAC) were predominantly high (64%) in this study. *Candida tropicalis* (44%) was the most common amongst NAC in this study, while *Candida albicans* was 32%.

SPECIES	NUMBER	PERCENTAGE %
C. albicans	18	36
C. tropicalis	22	44
C. parapsilosis	6	12
C. glabrata	4	8
total	50	100

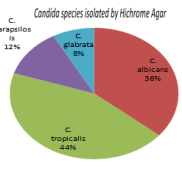
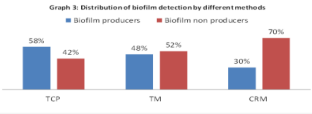


Table /Graph 2. Candida species isolated by Hichrome Agar

Table/Graph 3.Shows Out of all phenotypic methods for biofilm production TCP detected maximum number (58%) of biofilm producing candida spp. followed by Tube method 24 (48%) and Congo red agar method 15(30%).

Table 3: Distribution of biofilm detection by different methods

Biofilm	TCP	TM	CRM
Biofilm producers	29 (58%)	24 (48%)	15 (30%)
Biofilm non producers	21 (42%)	26 (52%)	35 (70%)
Total	50	50	50



Chi-square = 8.1241 with degree of freedom; P<0.05

Comparision of Sensitivity, Specificity, Positive predictive value and Negative predictive value of TM and CRM

Results	TM		CRA		P value
	No.	%	No.	%	
Sensitivity	23/29	79.31%	13/29	44.83%	<0.05
Specificity	20/21	95.24%	19/21	90.48%	0.548
PPV	23/24	95.83%	13/15	86.67%	0.2937
NPV	20/26	76.92%	19/35	54.29%	0.068

Table 5: Statistical results for TM and CRA taking TCP as gold standard.

*'Z' test for difference of two proportions

Considering TCP method as a gold standard for this study sensitivity data was compared with the data from TM and CRA method. Parameters like sensitivity, specificity, negative predictive value and positive predictive value were calculated. Significant difference was found in sensitivity of TM and CRA with P value <0.05 and in negative predictive value of TM and CRA with P value 0.164.

Table/Graph 4. Shows Biofilm production was observed more with *non-albicans Candida* species 19(66%) compared to *C. albicans* species 10(34%).

	number	Percentage%
C. albicans	10	34
C. Non albicans	19	66
Total	29	100

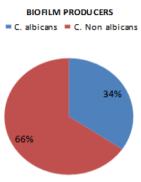


Table /Graph 4. Biofilm producers

Table 6. Among *non albicans candida species* biofilm production was seen maximum 16(84.21%) with *C. tropicalis.*

Table 6. Biofilm production by different *candida species*

		Tissue culture method				Tube method				Congo red method		
species	No.	strong	moderate	weak	negative	strong	moderate	weak	negative	strong	moderate	weak/ negative
C. tropicalis	22	12	4	1	5	5	7	3	7	4	5	13
C. albicans	18	6	4	0	8	3	5	2	8	5	1	12
C. parapsilosis	6	0	2	1	3	2	1	1	2	0	0	6
C. glabrata	4	0	1	0	3	0	1	1	2	0	0	4
total	50	18	11	2	19	10	14	7	19	9	6	35

DISCUSSION

In our study we found out of 50 candida species tested 29(58%) were found to be biofilm producer. Among biofilm positives, *Candida albicans* 55.55%, which was quite low as compared to non- albicans Candida, specially *C.tropicalis*, which gave 72.72% biofilm positives. In a study by Khatri S et al out of 80 candida species tested 49(61.3%) were found to be biofilm producers. Among biofilm positives, *Candida albicans* was 52.2%, which was quite low as compared to non- albicans Candida, specially *C.tropicalis*, which gave 70.27% biofilm positives [18]. Similarly, In a study by Golia S et al, Out of 108 Candida species tested 71(65.74%) were found to be biofilm producers. Biofilm production was found to occur most frequently among non-albicans Candida 44(61.97%) than *Candida albicans* 27(38.03%)[3]. Saurabh Muni et al also found that The biofilm positivity was found more with Non albicans Candida species (78.9%) as compared to *Candida albicans* (54.8%)[19].In our study , the sensitivity and specificity of CRA method and TM method was evaluated by using TCP method as a gold standard. In our study by TM method, we have got 24(48%) biofilm positives which includes both moderate and strongly positives, and 26 (52%) negative for biofilm. Statistical analysis in our study indicates that sensitivity and specificity of TM was 79.31% and 95.24% respectively taking TCP as gold standard. Similarly in study conducted by Khatri S. et al sensitivity and specificity of TM was 91.8% % and 100% respectively. This related well with our study as well where TM showed high sensitivity and specificity when compared to TCP method. And sensitivity and specificity of CRA in our study is 44.83% and 90.48% respectively which was found to lower than TM taking TCP as gold standard. Similarly in study conducted by Khatri S. et al sensitivity and specificity of CRA is 10.20% and 90.32% respectively. A low sensitivity of CRA does not favor its use as a screening method as Congo red binds to chitin and glucan and to extracellular matrix polysaccharide generated by *Candida*. However TM correlates well with TCP method for biofilm production with better sensitivity but it was difficult for TM to discriminate between moderate and weak biofilm isolates due to the variability is observed by different observers, So TM cannot be recommended as a general screening test to identify biofilm producing isolates.

Significance difference was found in sensitivity of TM and CRA with P value <0.05.

According to our results by TCP method, we conclude that the TCP method can be recommended as a general screening method for detection of biofilm formation of clinical Candida isolates in laboratories, as it allows an easy and quantitative as well as qualitative detection of biofim production.

CONCLUSION

The study demonstrates that biofilm formation and consequent infections of medical indwelling devices is a serious problem in hospitals in this era. Despite the large number of antimicrobial agents available, it is extremely difficult to eradicate microorganisms from biofilms as a high degree of resistance is demonstrated by most of organisms isolated. Our data indicates that the TCP is an accurate and reproducible method for screening and can serve as a reliable quantitative tool for determining biofilm formation by clinical isolates of Candida species. Accurate species identification is important for the treatment of Candida infections, as most of the non-albicans Candida are inherently resistant to anti-fungal agents especially to azoles. Hence rapid identification and speciation of Candida species is essential in guiding appropriate anti-fungal therapy. But further studies need to be done in this field to establish their role in infections.

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