Original Article

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# Comparison of Methods of Biofilm Detection in Urinary *Candida* Isolates and Evaluating its Role in Persistent Candiduria

Microbiology Section

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## ABSTRACT

**Introduction:** Candiduria is a common finding in catheterised patients with increasing trend of non albicans *Candida* species. Biofilm formation on indwelling catheters leads to persistent colonisation ending up in infection in immunocompromised patients.

**Aim:** This 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 and evaluating the role of biofilm in persistent candiduria.

**Materials and Methods:** A prospective study was conducted in Microbiology Department. Total 50 non repeat urine samples were selected from the patient's sample received in the laboratory routinely. Samples with significant number of pus cells and budding yeast cells in direct microscopy and significant count of *Candida* species in culture from catheterised patients were subjected to HiChrom agar for species identification and tube method, congo red agar and tissue culture plate method for biofilm detection. Diagnostic test analysis was done for the three biofilm detection methods taking tissue culture plate methods as gold standard. Chi-square analysis was performed for comparison of persistent candiduria with duration of catheterisation and biofilm.

**Results:** Sensitivity and specificity for tube method was 100% and 0% respectively and 26.53% and 100% respectively for Congo red agar. A significant difference was found in persistent candiduria in patients with duration of catheterisation of one week and those with more than one week ($\chi^2$ =0.0047 p-value=0.9) and also in proportion of biofilm positive *Candida* isolates in these two group of patients $\chi^2$=4.56, p-value=0.9).

**Conclusion:** Tube method showed excellent sensitivity while congo red agar had better specificity. This study shows that Candida colonisation and biofilm formation is associated with persistent candiduria.

**Keywords:** Catheter, Colonising, Hospitalised

## 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 a frequent finding in hospitalised patients with indwelling catheters,though its clinical significance is not yet established [3]. Shifting of trend to non-albicans *Candida* (NAC) infection with high antifungal resistance has been witnessed in last few decades [4-6]. Of major concern is isolation of *Candida* species in intensive care units responsible for increasing mortality in patients with co-morbidities [7]. Decision to treat such patients with anti-fungal drugs is complexed by understanding of the fact that there is a thin line between colonization and infection in patients in intensive care settings and drug resistance is major area of concern again in such patients.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 [8]. 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 [9]. Biofilm may form on any implantable device and majority of nosocomial infections are associated with biofilm infections of medical devices [10]. Biofilms of *Candida* species exhibits resistance to antifungal agents [11]. It is a major hidden challenge both for diagnosing in vitro susceptibility as well as therapeutic purposes. Several studies have been conducted showing *Candida* species isolation in different clinical samples, detection of biofilm by various methods and role of biofilm in antifungal resistance.

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 and evaluating the role of biofilm in persistent candiduria.

## MATERIALS AND METHODS

This prospective study was conducted in the Microbiology Department at Gobind Ballabh Pant Institute of Post graduate Medical Education and Research (GIPMER),

between the period of January 2017 to June 2017. During six month duration, a total of 1115 urine samples were received from catheterised patients admitted in various ICU, out of which candiduria was found in 19.28% (215) samples and 50 (23.25%) samples which showed repeated isolation of *Candida* were included in the study.

Urine samples received from patient with at least five days of catheterisation showing significant number of pus cells and budding yeast cells in direct microscopy and *Candida* species ($10^5$ CFU/mL) isolated after 24 hours of aerobic culture were included in the study

Urine samples received from uncatheterised patients and samples in which bacteria was isolated along with *Candida* species in culture were excluded from the study.

Repeat urine samples were asked from the 50 patients who fulfilled the inclusion criteria.

Persistent candiduria was defined as *Candida* species (≥$10^5$ CFU/mL) isolated in atleast two urine sample of a catherised patient with pyuria, collected at one week interval. Non persistent candiduria was defined as *Candida* species not isolated in repeat urine sample of a catheterised patient with pyuria. As per Hospital Infection surveillance data of this hospital,it is seen that *Candida* is repeatedly isolated in catheterised patients when urine culture is repeated at an interval of 7 -21 days. Therefore, the definition was set for study convenience. A detailed clinical history with emphasis on duration of catheterisation and underlying immune-suppressed conditions was taken. *Candida* isolated in any other sample including blood was also noted down from patient's clinical file. After screening the samples for pus and budding yeast cells by microscopy, urine sample were subjected to semi-quantitative culture on McConkey agar and 5% blood agar in 37° C for 24 hours. Colonies obtained were identified as Gram positive budding yeast cells by Gram stain.

**Germ Tube Test (GTT):** For preliminary differentiation of *Candida* albicans and NAC, a light suspension of the yeast colonies was made in 0.5 mL of serum in a sterile test tube and incubated for 2-3 hours in a 35-37°C incubator. A drop of the suspension was placed on a slide and examined under 40X for production of germ tubes. *Candida albicans* ATCC® 90028 was used as control strain. Isolates were stocked on Saboraud's Dextrose agar (SDA) with Chloramphenicol (5%) for further study

**Identification by HiChrom agar (HiMedia):** *Candida* isolates were subcultured on HiChrom agar (HiMedia) and incubated at 37°C as per manufacturer's instructions. *Candida albicans* ATCC®90028TM was used as control strain.

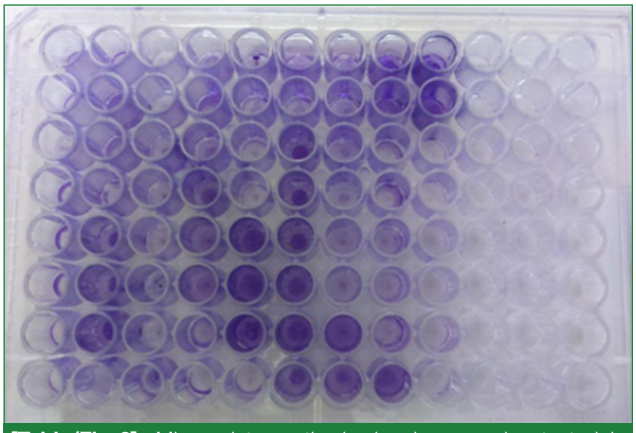
**Congo Red Agar (CRA) Method [12]:** Composition of the media is BHI (37 gm/L), glucose (80 gm/L), agar no.1 (10 gm/L) and Congo red stain (0.8 gm/L). Aqueous solution of Congo red was prepared and autoclaved separately and added to the agar after cooling to 55°. *Candida* strains were freshly inoculated from 24 hour growth on SDA plates. *Candida albicans* ATCC® 90028™ and *Candida parapsilosis* ATCC®96142™ served as controls for biofilm production. Positive result was indicated by production of black colonies and negative by pink colonies after 24-48 hours of aerobic incubation 37°C.

**Tube Method (TM) [12]:** A loop full of colonies from the surface of SDA plate was inoculated into polystyrene tube containing 10 mL of Saboraud's dextrose broth supplemented with glucose and incubated at 37°C for 48 hours. After broth aspiration, the tubes were washed once with distilled water and then stained with 1% safranin after media and yeast cells were discarded. All the samples were tested in triplicate. Grading was given negative, weak (1+), moderate (2+), strong (3+ and above) [Table/Fig-1].

**[Table/Fig-1]:** Results of Tube method.

**Microtiter Plate Method (MTP) [13]:** 100 µL aliquots of the cell suspension were filled in each well of a sterile, polystyrene, 96 well flat bottom microtitre plates The microtiter plates were incubated for 48-72 hours at 37°C. After incubation content of each well was gently removed by tapping the plates and stained with crystal violet (0.1% w/v). After rinsing the excess stain thoroughly and after proper drying, Optical Density (OD) of stained adherent *Candida* biofilm was determined with ELISA reader at wavelength of 492 nm. These OD values were considered as an index of *Candida* adhering to surface and forming biofilms. All the samples were tested in triplicate [Table/Fig-2].

Grading was given as: Strong (3+); Moderate (2+); Weak (1+) and negative as follows: If mean OD <0.120=non/weak biofilm formation; if mean OD is 0.120-0.240=moderate biofilm formation; if mean OD= strong biofilm formation.



**[Table/Fig-2]:** Micro plate method, showing samples tested in triplicate.

## STATISTICAL ANALYSIS

All statistical analysis were performed using Statistical Package for the Social Sciences (SPSS) version 12.0 Sensitivity, specificity, Negative Predictive Value (NPV) and Positive Predictive Value (PPV) were calculated for comparison of the three methods used for biofilm detection. Chi-square analysis was performed for comparison of difference in *Candida* isolation and biofilm formation with duration of catheterisation (p-value=0.9).

## RESULTS

Out of 50 patients in whom *Candida* species was isolated in urine, 42 (84%) patients were from ICU while only 8 (16%) were from ward.14 (28%) were female, while 36 (72%) were male. Majority of the patients (50%) were of age group 21-40.

The major co-morbid conditions associated with *Candida* isolation were use of mutiple antibiotics (100%), and diabetes (28%). Prophylactic antifungal was administered along with antibiotics in 6% patients [Table/Fig-3].

| Clinical Conditions | n (%) |
|---|---|
| More than one antibiotic | 50 (100) |
| Diabetes | 14 (28) |
| Steroid treatment | 4 (8) |
| Tuberculosis | 4 (8) |
| Prophylactic antifungal | 3 (6) |

**[Table/Fig-3]:** Co-morbid conditions associated with patients detection.

**Germ tube test:** 13 (26%) out of 50 showed production of germ tube. All the GTT positive *Candida* isolates were identified as *Candida albicans* by HiChrom agar.

**Identification by HiChrom Agar:** Non-albicans *Candida* (NAC) were predominantly high (74%) in this study. *Candida tropicalis* (54%) was the most common amongst NAC in this study, while *Candida albicans* was 26% [Table/Fig-4].

The average duration of catheterisation in patients with *Candida* isolation in urine sample was 15.7 days [Table/Fig-5]. *Candida* species was isolated after 5-7 days of catheterisation in 22 (44%) patients. Persistent candiduria was seen in 12 out of these 22 (54.54%) patients, and in 15 (53.57%) out of 28 patients in whom *Candida* species was isolated after 7 days of catheterisation. A significant difference was found in persistent candiduria in patients with duration of catheterisation of 5-7 days and those with catheterisation of more than one week ($\chi^2$=0.0047, p value=0.9).

| Species | Isolates (%) |
|---|---|
| *Candida tropicalis* | 27 (54) |
| *Candida albicans* | 13 (26) |
| *Candida haemulonii* | 2 (4) |
| *Candida glabrata* | 3 (6) |
| *Candida krusei* | 2 (4) |
| *Candida parapsilosis* | 2 (4) |
| *Candida rugosa* | 1 (2) |

**[Table/Fig-4]:** Species identification by HiChrome agar detection.

| Biofilm Formation by TCP | | | | | |
|---|---|---|---|---|---|
| Days of Cathet-erisation | Isolates (%) | Strong | Moderate | Weak | Negative |
| 5-7 | 22 (44) | 15 | 4 | 2 | 1 |
| 8-14 | 10 (20) | 5 | 3 | 2 | 0 |
| 15-21 | 9 (18) | 6 | 2 | 1 | 0 |
| 22-28 | 1 (2) | 1 | 0 | 0 | 0 |
| > 28 | 8 (16) | 6 | 2 | 0 | 0 |
| (15.7) Average | 50 | 33 | 11 | 5 | 1 |

**[Table/Fig-5]:** Duration of catheterisation of *Candida* species isolation versus biofilm formation.

| | TCP | | TM | | CRA | |
|---|---|---|---|---|---|---|
| | +ive | -ive | +ive | -ive | +ive | -ive |
| Weak | 5 (10%) | 1 (2%) | 5 (10%) | 0 | 13 (26%) | 37 (74%) |
| Moderate | 11 (22%) | - | 8 (16%) | - | - | - |

**[Table/Fig-6a]:** Comparison of three phenotypic methods for biofilm.
Note: +ive = Positive; -ive = Negative

| Results | TM | | CRA | |
|---|---|---|---|---|
| Sensitivity | 100% | 92.75%-100% | 26.53% | 14.95%-41.08% |
| Specificity | 0% | 92.75%-100% | 100% | 2.50%-100% |
| PPV | 98% | 98%-98% | 100% | 98%-98% |
| NPV | - | - | 2.70% | 2.29%-3.18% |

**[Table/Fig-6b]:** Statistical results for TM and CRA taking TCP as gold standard.

Biofilm production was positive in 21 (95.45%) out of 22 *Candida* species isolated from patients with 5-7 days of catheterisation. Out of these 21 *Candida* isolates, 15 (71.42%) were strong biofilm producers. 100% *Candida* species were positive for biofilm production in 28 patients in whom *Candida* species was isolated after one week of catheterisation and 18 (64.28%) of these 28 were strong biofilm producers. A significant difference was found in proportion of biofilm positive *Candida* isolates in patients catheterised for 5-7days and those catheterised for more than one week ($\chi^2$=4.56, p-value=0.9).

Out of the three methods evaluated for biofilm detection in *Candida* species, only 13 (26%) were positive for biofilm by CRA method. By TM,37 (78%) were strongly positive, 8 (16%) moderately positive and 5 (6%) weakly positive. By TCP method 33 (66%) were strongly positive,11 (22%) were moderately positive, 5 (10%) were weakly positive, while 1 (2%) were

| Species (n=50) | TCP | | | | TM | | | | CRA | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Strong | Moderate | Weak | Negative | Strong | Moderate | Weak | Negative | Positive | Negative |
| *C.tropicalis* (27) | 21 | 5 | 0 | 1 | 23 | 3 | 1 | 0 | 10 | 17 |
| *C.albicans* (13) | 7 | 5 | 1 | 0 | 8 | 5 | 0 | 0 | 3 | 10 |
| *C.glabrata* (3) | 0 | 1 | 2 | 0 | 1 | 0 | 2 | 0 | 0 | 3 |
| *C.haemulonii* (2) | 2 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 2 |
| *C.parapsilosis* (2) | 0 | 0 | 2 | 0 | 0 | 0 | 2 | 0 | 0 | 2 |
| *C.krusei* (2) | 2 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 2 |
| *C.rugosa* (1) | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 |
| Total | 33 | 11 | 5 | 1 | 37 | 8 | 5 | 0 | 13 | 27 |

**[Table/Fig-7]:** Species distribution versus biofilm production by three methods.

negative [Table/Fig-6a]. TM was 100% sensitive in detecting biofilm formation. CRA had 100% specificity but a poor sensitivity (26.53%) taking TCP as gold standard for detection of biofilm formation in *Candida* species [Table/Fig-6b].

[Table/Fig-7] shows by TCP method, major strong biofilm producers were *Candida tropicalis* (63.63%) followed by *Candida albicans* (21.21%). It can be said that the sensitivity of TCP is more for NAC, *Candida tropicalis* was the only isolate to show negative results by this method.

[Table/Fig-8,9] shows biofilm production and *Candida* species distribution in samples with non persistent candiduria and samples with persistent candiduria respectively. There was no difference noticed in grade of biofilm production and

| BF Production | Catherisation Duration | n | 23 | |
|---|---|---|---|---|
| Strong | 5-7 days=3 | 11 | C.t* | 9 |
| | 8-14 days=2 | | | |
| | 15-21 days=3 | | C.k** | 1 |
| | >28 days=3 | | C.r*** | 1 |
| Moderate | 5-7 days=3 | 8 | C.t* | 3 |
| | 8-14 days=2 | | C.a# | 5 |
| | 15-21 days=1 | | | |
| | 21-28=1 | | | |
| | >28 days=1 | | | |
| Weak | 5-7 days=2 | 3 | C.a# | 1 |
| | 15-21 days=1 | | C.g## | 1 |
| | | | C.p### | 1 |
| Negative | 15 days | 1 | C.t* | 1 |
| ***Candida* isolated in any other sample simultaneously** | | | | |
| Sample | n | BF$ positivity in urinary isolates | | |
| Blood | 1 | Strong | | |

**[Table/Fig-8]:** Detailed information of samples with non-persistent candiduria.
Note: * *C.tropicalis*, ** *C.krusei*, *** *Candida rugosa*, # *Candida albicans*, ## *Candida glabrata*, ### *Candida parapsilosis*, n=number, $ Biofilm

| Biofilm Production | | n | Species (n=27) | |
|---|---|---|---|---|
| Strong | 5-7 days=11 | 22 | C.t* | 12 |
| | 8-14 days=2 | | C.h*** | 2 |
| | 14-21 days=5 | | C.a# | 7 |
| | >28 days=4 | | C.k** | 1 |
| Moderate | 5-7 days=1 | 3 | C.t* | 2 |
| | 8-14 days=1 | | C.g## | 1 |
| | >28 days=1 | | | |
| Weak | 8-14 days=2 | 2 | C.p### | 1 |
| | | | C.g### | 1 |
| ***Candida* isolated in any other sample simultaneously** | | | | |
| Sample | | n | BF Positivity in urinary isolates | |
| Blood with/without other sample | | 7 | All strong | |
| Pus | | 1 | Moderate | |

**[Table/Fig-9]:** Detailed information of samples with persistent candiduria.
Note: * *C.tropicalis*, ** *C.krusei*, *** *Candida rugosa*, # *Candida albicans*, ## *Candida glabrata*, ### *Candida parapsilosis*, n=number, $ Biofilm

species distribution in both the groups. Out of 27 patients of persistent candiduria, 8 patients had concomitant *Candida* isolation in other clinical sample. 7 (87.5%) out of 8 patients had concomitant candidemia. *Candida tropicalis* was isolated in three consecutive urine samples in 3 (11.11% ) out of 27 patients with moderate to strong biofilm production. *Candida* species was also isolated twice in blood culture from 2 out of these 3 (66.67%) patients, when collected within one week interval. While contrastingly, in the other group, only one patient had concomitant *Candida* isolation in blood sample.

## DISCUSSION

In this study, rate of *Candida* species isolation from ICU patients (84%) was similar to that of Khatri S et al., who reported 66.25% isolation from ICU patients [14]. Predominant isolation of NAC in the present study (74%) was similar to other

studies [6,8,9,14,15],except one study where higher number of *Candida albicans* was reported [16]. Pre disposing factors in this study like use of multiple antibiotics (100%), diabetes (28%), were similar to those reported earlier [3,14,16,17]. In this study, three patients were on prophylactic antifungals and all the three patients had *Candida tropicalis* isolated in their urine sample. This finding supports the fact that use of prophylactic broad spectrum antifungal in ICU patients leads to colonisation or infection with NAC [18,19].

A single episode of candiduria in a voided midstream urine sample needs to be correlated clinically as in most patients, candiduria merely represent colonisation. Catheterised patients with significant candiduria without bacteriuria were included in the study. To rule out colonisation, a repeat urine sample collected atleast seven days after or collected during change of catheter (whichever first) was requested. The biofilm production in *Candida* species isolated from urine of patients with persistent candiduria and patients with non-persistent candiduria was compared. Only first isolate of each patient was included in the study.

Twenty two (44%) *Candida* isolation was within 5-7 days of catheterisation out of which 12 (54.54%) were of persistent candiduria. Persistent candiduria was also found in 15 out of 28 (535.7%) patients in whom *Candida* species was isolated after one week of catheterisation. A significant difference was found in persistent candiduria with duration of catheterisation and also in biofilm formation with duration of catheterisation. This is a novel finding in this study, and to the best of knowledge, has not been reported earlier This finding shows that with increased duration of catheterisation, colonising flora of candiduria becomes persistent and by producing biofilm slowly contributes to morbidity of the patient by causing UTI.

In this study, three phenotypic methods were evaluated for detection of biofilm in *Candida* species. TCP had 98% positivity; CRA method gave the minimum positive results (13%), while TM showed positive results in 100% samples. A positivity rate of 11.76%-34% by TCP has been reported earlier [8,20]. Similar results were reported by Khatri S et al., [14]. A low sensitivity of CRA in this study 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* [8]. It has however been reported as a reliable method for screening of biofilm formation with 38.33% positivity in muco-cutaneous samples [9].TM showed 100% sensitivity and 0% specificity [Table/Fig-6b]. Khatri S et al., reported a sensitivity and specificity of 91.8% and 100%, respectively for TM [14]. The author had included the weakly positive isolates as negative. Few other studies reported about 63-66% positivity by TM [6,21]. Specificity of TM in the present study might have been effected by subjective error and counting weakly positive as positive. Few studies have reported TM as a reliable method [8,15]. This method can be used to screen biofilm formation in *Candida* species as it is a simple and fast method with no extra chemicals or equipments required yet its interpretation is subjective. However, positive results by this method must be confirmed by other specific methods.

Sample type selection for evaluation of biofilm detection methods by *Candida* species has been found to differ in different studies. Few studies have shown that results for a particular method vary with type of clinical sample tested [4,15]. Highest biofilm positivity has been reported in bloodstream isolates [21], in vaginal swabs and urine isolates [4] and least in respiratory tract [21]. Thus, the role of biofilm as a virulent factor varies in relation to type, site and stage of infection. It appears to contribute most in pathogenesis of UTI and other luminal infections, compared to other clinical conditions. Biofilm studies are often done on *Candida* strains may not represent BF formation in clinical strains as they have been passage in laboratories and adapted to culture media [4]. Higher biofilm formation on NAC in this study was in concordance with other studies [4,6,14,15,21]. Dag I et al., claimed that *Candida albican*s had slightly more percentage of biofilm positivity (39.3%) with respect to NAC (37.7%) [12].

In this study, *Candida tropicalis* (63.63%) mainly contributed to the strong biofilm producers which was similar to an earlier report where MTP was used [4]. Another author reported *Candida krusei* and *Candida tropicalis* as strong biofilm producers, *Candida albicans* as weak biofilm producers by CRA method [9].In contrast, *Candida parapsilosis*, *Candida pseudotropicalis*, and *Candida glabrata* has been reported to produce significantly less biofilm production than *Candida albicans* [22].

Stronger biofilm producers were comparatively more in patient with persistent candiduria. Candidemia was also more common in patient with persistent candiduria [Table/Fig-8,9]. Repeated *Candida* isolation in three urine sample of three patients shows that persistence of infection is directly related to stronger biofilm production. Biofilm production was not done for isolates from other clinical samples of these patients, so co relation of biofilm production in candidaemia could not be shown. A molecular typing of strain isolated from each sample of a given patient could not be done. In a study, comparison and identification of each strain of *Candida* isolated from different site of patients showed that biofilm is a stable characteristic of *Candida* strain that is affected by the chronicity of infection [4]. Catheter associated urinary biofilms can actually mislead results of microbiology studies, both in terms of the species identified and their susceptibilities as they will depict the results of only the free-floating organisms at the time of urine collection [23].

## LIMITATION

A low sample size might have affected the results of biofilm. Biofilm detection of *Candida* isolated from other samples and molecular typing could not be done.

## CONCLUSION

In the present study, predominant NAC has been found in urine sample of catheterised patients in ICU. CRA is an unreliable method while TM is sensitive though non specific method. Increased duration of catheterisation is associated with persistent candiduria and biofilm formation.

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