Original Article

# Characterization of fungal biofilm-based catheter-related sepsis


## Abstract

**Background:** Fungi most commonly associated with nosocomial septicemia are in the genus *Candida*. Attachment of yeasts to intravascular surfaces is the crucial step in initiating colonization by yeast cells, forming biofilms and resulting in disseminated infection depending on various factors. **Aims:** To study the rate and profile of fungal biofilms in catheter-related sepsis (CRS) and antifungal resistance among the clinical isolates of CRS was the aim of this study. **Materials & Methods:** In all, 135 hospitalized pediatric patients with peripheral intravascular catheters (IVCs) and clinical suspicion of nosocomial septicemia were studied. The yeast isolates causing CRS were identified and characterized, and antifungal susceptibility testing by microplate alamar blue method (minimum inhibitory concentration) was also done. The fungal biofilm formations were visualized by scanning electron microscopy and tube method. **Results:** 7.4% patients with IVC had CRS, majority being caused by *Candida albicans* biofilms. *In vitro* antifungal susceptibility testing of yeast isolates causing CRS demonstrated moderate to high level of resistance to fluconazole (70%). Voriconazole was the most optimum drug to cure such infections. **Conclusion:** This study illustrates the need for exploration of biofilm-based CRS (fungemia) in hospitalized patients and to design practical guidelines for their management (diagnosis and treatment).



**Key words:**
*Catheter-related sepsis, fungal biofilm, scanning electron microscopy*


## Introduction

Peripheral intravascular catheters (IVCs) play an inevitable part in management of hospitalized patients in modern-day medical practice. The wide use of IVCs in hospitalized patient also makes them prone to biofilm colonization and further leads to nosocomial septicemia. Most nosocomial bloodstream infections among pediatric patients are related to the use of IVCs.[1] By and large, medical devices installed in patients are colonized by microbes which often organize themselves on device surface in form of biofilms. Biofilms are consortium of microbes which are embedded within a matrix of extracellular polymeric material and display an altered phenotype. Biofilms act as threat for persistent infection and also make therapeutic measures refractory.[2,3] Fungal implant infections are less common than bacterial infections but tend to be more serious and are an increasing problem.[4] Among fungi, *Candida* spp. is most common pathogen for such type of infections.[4] These organisms produce biofilm on synthetic materials, which not only facilitates colonization of the organisms to devices but also leads to resistant nosocomial infections.

Demonstration of biofilm formation on medical devices often needs specialized microscopy facilities. Scanning electron microscopy (SEM), because of its high-resolution properties, has been used widely by biofilm scientists for examination and characterization of biofilms on medical devices.[5] Confocal scanning laser microscopy and fluorescence microscopy are the other two recently used


| Access this article online | |
|---|---|
| Website: http://www.cysonline.org | Quick Response Code |
| DOI: 10.4103/2229-5186.94317 | |



**Monil Singhai, Abida Malik[1], Mohd. Shahid[1], Mohd. Ashraf Malik[2], Rajeev Goyal[3]**

*Department of Microbiology, Govt. Medical College, Haldwani, Uttarakhand, [1]Jawaharlal Nehru Medical College, AMU, Aligarh, [2]Department of Pediatrics, Jawaharlal Nehru Medical College, AMU, Aligarh, [3]Department of Biochemistry, Govt. Medical College, Haldwani, Uttarakhand, India*

**Address for correspondence:**
Dr. Monil Singhai,
Assistant Professor, Department of Microbiology,
Govt. Medical College, Haldwani, Uttarakhand - 263 139, India.
E-mail: drmonil@gmail.com

methods to visualize elaborate structures of biofilms, which require a suite of fluorescent stains.[5] Using these techniques, biofilms have been shown to consist of matrix-enclosed microcolonies. The use of specialized microscope is, however, costly and limited as a research tool. Recently, tube method and tissue culture plate method have been used by some workers to demonstrate biofilm formation, which is not only cheaper technique but also simple to perform.[6-8]

Progression of catheter colonization by biofilms to symptomatic bloodstream infection is probably a quantitative and universal phenomenon. The microbial colony counts (≥15 CFU) or (≥1000 CFU) recovered by the semiquantitative or quantitatitive catheter cultures, respectively, are most reliable techniques for diagnosis of catheter-related sepsis (CRS).[9,10] CRS can be defined as "bacteremia or fungemia in a patient with an intravascular device, with one or more positive blood cultures obtained by peripheral vein and clinical manifestations of sepsis in the absence of any source of sepsis apart from the device with semiquantitative (>15 cfu) culture from a catheter segment, and the same organism obtained by peripheral culture" as per guidelines given by Infectious Diseases Society of America.[10]

Management of biofilm-based device related infection is often challenging. Microbes express an altered phenotype in biofilm, which confer an inherent resistance to even most effective antimicrobial agents. The most commonly used antifungal agent for treating infections caused by *Candida* spp. is fluconazole. The cellular target of fluconazole and other azole derivatives in *Candida* spp. is cytochrome P-450, and mutations in it can contribute to even cross resistance in *Candida* spp.[11]

This study was conducted in patients with IVCs with the following aim and objectives (1) to find out the rate of CRS caused by fungal biofilms and (2) to study the antifungal resistance in them.

## Materials and Methods

A prospective study was carried out on 135 hospitalized pediatric patients with peripheral IVCs, who were clinically suspected of nosocomial septicemia. This study was conducted after taking permission from institutional ethical committee.

Inclusion criterions were as follows:
- Age group (0–14 years)
- Clinical signs of systemic infection in patient (e.g., fever, chills, and/or hypotension) developed after 48 hours of admission.

Exclusion criterion was as follows:
- Other sources of septicemia present (e.g. infusate related, catheter hub related, endogenous).

Intravascular catheter tips were taken in universal sterile container on clinical suspicion of nosocomial septicemia. Blood samples of the same patient were also collected at the time of withdrawal of intravascular catheter and after 2 days. All samples were collected under complete aseptic conditions and transported immediately to the microbiology laboratory for processing. Hemogram (TLC, DLC, and ESR), blood, urine culture, etc. were also done at the time of admission to rule out any infective etiology and as and when required.

Semiquantitative catheter culture by roll plate method was done on Sabouraud's Dextrose Agar (SDA) plates, one each being kept at 25°C and 37°C, respectively.[9] Blood was directly collected by venipuncture and inoculated in two biphasic blood culture bottle (BHI agar and broth), one each being kept at 25°C and 37°C, respectively.

The fungal isolates obtained from IVCs and corresponding blood cultures were identified and characterized. The basis of identification was their colony characters, germ tube production, morphology on cornmeal agar, sugar fermentation tests, sugar assimilation tests, and chromagar.[12]

The segment was then immersed in 1% glutaraldehyde to be used for SEM to confirm yeast biofilms on their surface only if semiquantitative catheter culture yielded ≥15 CFU of fungal isolates.

### Detection of biofilms

#### *Scanning electron microscopy*

The catheter segments were rinsed in 0.1 M phosphate buffer and then placed in 1% Zetterquist's osmium for 30 min. The segment was subsequently dehydrated in a series of ethanol washes (70% for 10 min, 95% for 10 min, and 100% for 20 min), treated (two times, 5 min each) with hexamethyldisilizane (Polysciences Inc., Warrington, PA), and finally air-dried in a desiccator. The segment was coated with gold-palladium (40%/60%). After processing, segment was observed with a scanning electron microscope (Leo 435 VP) in high vacuum mode at 15 kV. The images were processed for display using Photoshop software (Adobe Systems Inc., Mountain View, CA).

#### *Tube method*

Biofilm formation ability of yeast isolates obtained both from catheter culture and corresponding blood cultures was also tested by tube method, as described by others with slight modification.[7,8] Briefly, 0.5 ml ($1.5\times10^8$ organism/ml) of 48-hour culture saline washed suspension was inoculated into a polystyrene tube containing 4.5 ml of Luria–Bertani broth. Tubes were incubated at 37°C for 24 hours without agitation. After 48 hours, the culture broth in the tube was aspirated, and tubes were washed twice with distilled water. The walls of the tube were stained with Crystal

violet after media and yeast cells were discarded. Biofilm formation was considered positive when a visible film lined the wall and bottom of the tube. Ring formation at the liquid interface was not indicative of biofilm formation. The adherent layer of biofilm forming isolates in tubes was scored as negative, weak (1+), moderate (2+ or 3+), or strong (4+). Each isolate was tested at least three times and read independently by two different observers. Strong biofilm producer *Staphylococcus epidermidis* ATCC 35984 and non-biofilm producer *Candida albicans* ATCC 10231 were used as a positive and negative control, respectively.

### *Antifungal susceptibility testing*

Antifungal susceptibility testing for 10 biofilm-producing yeast isolates was performed by the broth microdilution method as per Clinical and laboratory Standard Institute (CLSI) complementing with use of alamar blue, a cell viability indicator as follows.

### *Microplate alamar blue method*

Microplate alamar blue method is a simple, reproducible method for determining *in vitro* drug susceptibility of antifungals active against yeast biofilms.[13] Alamar blue/resaruzin developed by (Alamar Biosciences, Inc., Sacramento, CA) is an oxidation–reduction indicator in which a color change occurs in response to chemical reduction in the growth medium by the growing organisms. The extent of reduction is a reflection of cell viability. The indicator can be read visually or with a fluorometer. Standard antifungal powders of fluconazole (HiMedia), ketoconazole (HiMedia), itraconazole (HiMedia), and voriconazole (Pfizer) were obtained from the respective manufacturers. Stock solutions were prepared in dimethyl sulfoxide (voriconazole) and water (fluconazole, ketoconazole, itraconazole). Two-fold serial dilutions were prepared exactly, and minimum inhibitory concentration (MIC) was interpreted as outlined in CLSI [Table 1].[14]

Briefly, biofilm was grown in a 96-well u bottom polystrene plate; 90 µl (SDA broth with 8% glucose) + 10 µl of tested strains in 0.9% NaCl ($1.5\times10^3$ cells/ml) were added to the wells and cultivated for 2 days at 30°C. Wells were subsequently washed thrice to remove planktonic cells and then were exposed to 100 µl various drug concentrations with 0.001% alamar blue. Total volume was 200 µl per well. Final concentrations of antifungal agents tested were 0.125–256 µg/ml. The viability of the biofilm was assessed by reduction of alamar blue after 2 days at 37°C. MIC was determined by the concentration in the well that showed 90% inhibition of change. Drug-free, yeast-free controls, one reference isolate *C. albicans* (ATCC 90028) and two quality control isolates *Candida parapsilosis* (ATCC 22019), *Candida krusei* (ATCC 6258), were also tested every time a set of isolates was evaluated.

**Table 1: Interpretative guidelines for susceptibility testing *in vitro* for yeasts**

| Antifungal agents | Susceptible (S) MIC (µg/ml) | Susceptible dose-dependent (S-DD) MIC (µg/ml) | Resistant (R) MIC (µg/ml) |
|---|---|---|---|
| Fluconazole | ≤8 | 16–32 | ≥64 |
| Ketoconazole | ≤0.0625 | 0.125–0.5 | ≥1 |
| Itraconazole | ≤0.125 | 0.25–0.5 | ≥1 |
| Voriconazole | ≤0.5 | 1–2 | ≥4 |

MIC – Minimum inhibitory concentration

### Establishment of biofilm-based CRS

Whenever semiquantitative catheter culture yielded ≥15 CFU of fungal isolates with biofilm formation ability, it was suggestive of biofilm-based CRS. The accompanying signs of systemic infection in patient (e.g., fever, chills, and/or hypotension), no other source of infection present (e.g., infusate related, catheter hub culture), and corresponding blood cultures positivity with the same organism (species and antifungal susceptibility) confirmed biofilm-based CRS.

## Results

In all, 10 out of 135 (7.4% patients with IVCs) had biofilm-based CRS (fungemia). Seven patients had infection due to *C. albicans*, and three were due to *C. krusei*, *Candida gullermondii*, and *Geotrichum* spp. each. SEM findings showed biofilm formation in catheters of all the patients with culture yield ≥15 CFU fungal isolates The useful information on the different cellular morphologies present on the catheter surface were obtained on scanning microscopy. Mature biofilms consisted of a dense network of cells of all morphologies (yeast, pseudohyphae), deeply embedded in a matrix consisting of extracellular slime substance [Figure 1]. All yeast isolate obtained from catheter and corresponding blood culture associated with CRS showed 4+ (strong biofilm production).

### Determination of *in vitro* susceptibilities of yeast isolates to various antifungal agents

Minimum inhibitory concentration (MIC) profiles of yeast isolates causing biofilm-based CRS were determined by microplate alamar blue method, as shown in [Table 2]. *In vitro* antifungal susceptibility testing of yeast isolates causing CRS demonstrated moderate (SDD) to high (R) level of resistance to fluconazole (70%) followed by ketoconazole (50%) and itraconazole (40%). Voriconazole was the most susceptible drug found with only 30% moderate resistance.

## Discussion

Catheter colonization can occur even within 24 hours of installation.[2] The catheters are colonized typically by organisms which comprise natural flora surrounding the site of catheter insertion. Initially microbes may contaminate

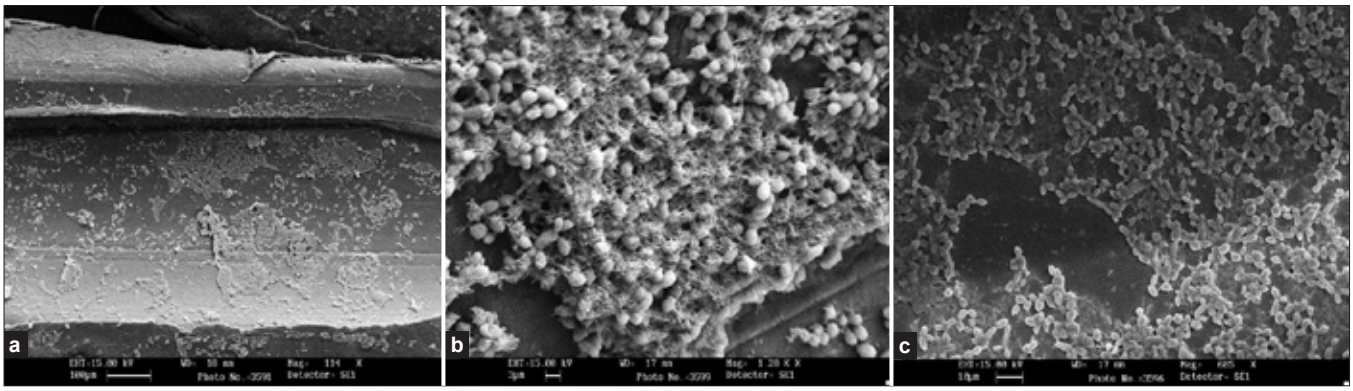

**Figure 1:** *Candida albicans* (a,b) and *Candida krusei* biofilms (c) demonstrated on intravascular catheter of pediatric patient as visualized on SEM

**Table 2: MIC profiles* (μg/ml) of biofilm-forming yeast isolates and reference strains to various antifungal agents (*n*=10)**

| Stain no./antifungals (MIC) | Isolates | Fluconazole | Ketoconazole | Itraconazole | Voriconazole |
|---|---|---|---|---|---|
| 1) | *Candida albicans* | ≥64 (R) | 16 (R) | 2 (R) | 2 (S-DD) |
| 2) | *Candida albicans* | ≥64 (R) | 8 (R) | 1 (R) | 1 (S-DD) |
| 3) | *Candida krusei* | ≥64 (R) | 2 (R) | 0.25 (S-DD) | 1 (S-DD) |
| 4) | *Candida albicans* | ≥64 (R) | 0.5 (S-DD) | 0.25 (S-DD) | 0.5 (S) |
| 5) | *Geotrichum spp.* | ≥64 (R) | ≤0.0625 (S) | 0.125 (S) | 0.5 (S) |
| 6) | *Candida albicans* | 16 (S-DD) | ≤0.0625 (S) | ≤0.125 (S) | 0.25 (S) |
| 7) | *Candida gullermondii* | 8 (S-DD) | 0.25 (S-DD) | ≤0.0625 (S) | 0.25 (S) |
| 8) | *Candida albicans* | 4 (S) | ≤0.0625 (S) | 0.125 (S) | 0.25 (S) |
| 9) | *Candida albicans* | 4 (S) | ≤0.0625 (S) | ≤0.0625 (S) | ≤0.125 (S) |
| 10) | *Candida albicans* | 2 (S) | ≤0.0625 (S) | ≤0.0625 (S) | ≤0.125 (S) |
| 11) | *C. albicans* (ATCC 90028) | 0.5 | – | – | – |
| 12) | *C. parapsilosis* (ATCC 22019) | 2 | ≤0.0625 | ≤0.0625 | ≤0.125 |
| 13) | *C. krusei* (ATCC 6258) | 32 | 0.25 | 0.125 | 0.125 |

*(R) – Resistant; (S) – Sensitive; (S-DD) – Susceptible dose-dependent (*n* = 10)

the catheter on its outer surface, later on microbes adhere on the surface of catheter and track along with the catheter when it is tunneled to its appropriate destination. This often results in biofilm-based CRS.[15] SEM has the capacity to visualize complex images and offers excellent resolution properties necessary to confirm biofilms and to study their detailed architecture. But due to high cost of instrument and specialized technique required for visualization, its role has been restricted in our country. The tube test correlates well with other techniques for strongly biofilm-producing isolates, but its results are difficult to interpret in cases of weak and non-biofilm-producing isolates as confirmed in some studies.[7,8] In our study, all yeast isolates were strong biofilm producers consequently fully correlated tube method for testing biofilm formation with microscopy findings. Thus, this method can be commonly used in clinical practice to demonstrate biofilms where sophisticated microscopy techniques are not available.

In our study, 7.4% patients with IVCs had CRS due to fungal biofilms, which is quite alarming. A similar study from India has reported slightly higher colonization rates (12.5%) by *Candida* spp.,[16] although they did not trace its progression to CRS. This finding in our study not only highlights the role of fungal biofilms in colonization but also signifies its progression to CRS. *Candida* septicemias (mostly by *C. albicans*) now represent approximately 10% of all nosocomial bloodstream infections and are usually catheter related.[10] *C. albicans* biofilms causing CRS were commonest in our study. Biofilms of non-*albicans Candida* spp., such as *C. krusei*, *C. gulleirmondii*, and *Geotrichum* causing CRS were also found. Non-*albicans Candida* was found to cause 3% CRS in one study.[17]

Fungal biofilm-based infections are common cause of morbidity in hospitalized patients, but no standardized method has been accepted by CLSI for antifungal susceptibility testing of biofilm-producing isolates. Broth microdilution method modified by use of colorimetric indicators such as alamar blue and tetrazolium reduction assay are few appropriate and convenient methods for detection of antifungal resistance in biofilm-producing isolates.[13,18] We used microplate alamar blue method for susceptibility testing of biofilm-producing isolates. Biofilms are basically formed after adherence to the surface, and

cell-wall-associated markers play an important role in the adherence to the surface, therefore cell-wall-inhibiting drug should be the choice of drug in such infections. Antifungal treatment strategies for fungal biofilms are limited to very few available options such as azoles, polyene macrolide (amphotericin B) and echinocandins. Among them, echinocandins (caspofungin acetate, micafungin, and anidulafungin) have emerged as breakthrough in treating fungal infections; however, they are still not widely used in our settings due to high cost. Therefore, azoles, although prone to resistance, are the only antifungal drugs available, other than amphotericin B, to treat systemic infections. Azoles, being safer than amphotericin B, are first choice of drug, for which susceptibility testing was done in our study. High and concomitant resistance of azoles among biofilm producers was found in our study. Such a finding in fungal biofilm isolates can be due to certain genetic mechanisms and needs to be explored at genetic level.[11,19] *In vitro*, *Candida* biofilms are highly resistant to most antifungal agents, thereby posing a therapeutic challenge in managing catheter-associated *Candida* spp. infections.[18] The high prevalence of CRS due to *Candida* spp. biofilms with high resistance to fluconazole in our study is in accordance with other studies in North Indian hospitalized children. [20,21] Voriconazole was the most effective drug to cure such infections in our setting.

This study also emphasizes that biofilm formation is a universal trait, and the extent and location of biofilm formation depends on various factors which needs to be investigated further. It can also be concluded that CRS by fungal biofilms are not only difficult to diagnose but also lead to resistant infections. The strong adherent character by virtue of biofilm-producing ability of such isolates on catheters makes them a persistent source of infection. Moreover, high antifungal resistance among azole groups in such infections necessitates exploring other therapeutic options such as echinocandins in our country. This study illustrates the need for exploration of biofilm-based CRS (fungemia) in hospitalized patients and to design practical guidelines for their management.

## Acknowledgment

Scanning electron microscopy was done by kind and generous support by sophisticated analytical instrumentation facility (DST), Department of Anatomy, All India Institute of Medical Sciences, New Delhi, India.

## References

1. Jarvis WR, Edwards JR, Culver DH, Hughes JM, Horan T, Emori TG, *et al*. Nosocomial infection rates in adult and pediatric intensive care units in the United States. Am J Med 1991;91(Suppl 3B):185S-91S.
2. Costerton JW, Lewandowski Z, Caldwell DE, Korber DR, Lappin-Scott HM. Microbial biofilms. Annu Rev Microbiol 1995;49:711-45.
3. Mah TF, O'Toole GA. Mechanisms of biofilm resistance to antimicrobial agents. Trends Microbiol 2001;9:34-9.
4. Fridkin SK, Jarvis WR. Epidemiology of nosocomial fungal infections. Clin Microbiol Rev 1996;9:499-511.
5. Ramage G, VandeWalle K, Wickes BL, Lopez-Ribot JL. Characteristics of biofilm formation by *Candida albicans*. Rev Iberoam Micol 2001;18: 163-70.
6. O'Toole GA, Pratt LA, Watnick PI, Newman DK, Weaver VB, Kolter R. Genetic approaches to study biofilms, In methods in enzymology: In: Ron J. Doyle Editor. San Diego: Academic Press; 1999;310:91-109.
7. Mathur T, Singhal S, Khan S, Upadhyay DJ, Fatma T, Rattan A. Detection of biofilm formation among the clinical isolates of *Staphylococci*: An evaluation of three different screening methods. Indian J Med Microbiol 2006;24:25-9.
8. Christensen GD, Simpson WA, Bisno AL, Beachey EH. Adherence of slime–producing strains of *Staphylococcus epidermidis* to smooth surfaces. Infect Immun 1982;37:318-26.
9. Maki DG, Weise C, Sarafin H. A semiquantitative culture method for identifying intravenous-catheter-related infection. N Eng J Med 1977; 296:1305-9.
10. Mermel LA, Allon M, Bouza E, Craven DE, Flynn P, O'Grady NP, *et al*. Clinical practice guidelines for the diagnosis and management of intravascular catheter-related infection: 2009 Update by the Infectious Diseases Society of America. Clin Infect Dis 2009;49:1-45.
11. Marichal P, Koymans L, Willemsens S, Bellens D, Verhasselt P, Luyten W, *et al*. Contribution of mutations in the cytochrome P450 14α-demethylase (Erg11p, Cyp51p) to azole resistance in *Candida albicans*. Microbiology 1999;145:2701-13.
12. Chander J. Candidiasis. Textbook of Medical Mycology, 2nd ed. New Delhi: Mehta Publishers; 2002. p. 212-27.
13. Repp KK, Menor SA, Pettit RK. Microplate alamar blue assay for susceptibility testing of *Candida albicans* biofilms. Med Mycol 2007;45:603-7.
14. Clinical and laboratory Standard Institute. Reference method for broth dilution antifungal susceptibility testing of yeasts: Approved standard. Approved Standard 2nd edn. Wayne, PA: National Committee for Clinical Laboratory Standards; 2007 Clinical and laboratory Standard Institute Document M27-A2, 2007.
15. Hawser SP, Douglas LJ. Biofilm formation by *Candida* species on the surface of catheter materials *in vitro*. Infect Immun 2005;62:915-21.
16. Subba Rao SD, Joseph MP, Lavi R, Macaden R. Infections related to vascular catheters in a pediatric intensive care unit. Indian Pediatr 2005;42:667-72.
17. Saxen H, Virtanen M, Carlson P, Hoppu K, Pohjavuori M, Vaara M, *et al*. Neonatal *Candida parapsilosis* with a high case fatality rate. Pediatr Infect Dis J 1995;14:776-81.
18. Ramage G, Walle KV, Wickes BL, López-Ribot JL. Standardized method for *in vitro* Antifungal susceptibility testing of *Candida albicans* biofilms. Antimicrob Agents Chemother 2001;45:2475-9.
19. Kojic EM, Darouiche RO. *Candida* infections of medical devices. Clin Microbiol Rev 2004;17:255-67.
20. Saha R, Shukla DD, Kumar A, Kaur IR. Pattern of *Candida* isolates in hospitalized children. Indian J Pediatr 2008;75:858-60.
21. Agarwal J, Bansal S, Malik GK, Jain A. Trends in neonatal septicemia: Emergence of non albicans *Candida* Indian J Pediatr 2004;41:712-5.

**How to cite this article:** Singhai M, Malik A, Shahid M, Malik MA, Goyal R. Characterization of fungal biofilm-based catheter-related sepsis. Chron Young Sci 2012;3:48-52.

**Source of Support:** Nil, **Conflict of Interest:** None declared