# Evaluation of polystyrene petri dish-based method for assessing biofilm formation *in vitro* by *Trichosporon* spp. and its comparison with test-tube method

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## ABSTRACT

**Background:** Microorganisms growing in a biofilm are associated with chronic and recurrent human infections and are highly resistant to antimicrobial agents. There are various methods to detect biofilm production such as tube method (TM) and tissue culture plate method followed by microscopic examination by inverted microscope. **Objective:** This study was conducted to compare two methods for the detection of biofilms. **Methods:** In this study, biofilm formation of ten isolates of *Trichosporon* spp. by test TM (TTM) and polystyrene petri dish method (PDM) was compared. **Results:** In the TTM, they were weak biofilm producers as compared to PDM where they were strong biofilms producers. **Conclusion:** PDM can be safely used to find out pattern of biofilm formation by *Trichosporon*.

**Key words:** *Normal saline, Peptone water dextrose, Polystyrene petri dish, Trichosporon, Safranine, Test tube*

Biofilms are defined as microbially derived sessile communities characterized by cells that are irreversibly attached to substratum or to each other. They are embedded in a matrix of extracellular polymeric substances and exhibit an altered phenotype with respect to growth rate and gene transcription [1]. Infections with invasive *Trichosporon* spp. are usually associated with central venous catheters, vesical catheters, and peritoneal catheter-related devices. The ability to adhere to and form biofilms on implanted devices can account for the progress of invasive trichosporonosis as it can promote the escape from antifungal drugs and host immune responses. Di Bonaventura et al. were the first to use electron microscopy to analyze the kinetics of biofilm formation and development on polystyrene surfaces. *Trichosporon* spp. is a genus of anamorphic yeasts (Basidiomycota, Hymenomycetes, and Trichosporonales) with distinct morphological characters of budding cells and true mycelium that disarticulates to form arthroconidia [2]. It is present in the external environment and is isolated mainly from the soil. It may also be present in water, air, and organic substrata [3]. In our body, populations of microbes such as yeasts form part of normal healthy human flora. *Candida* spp. is the fourth most commonly reported agent of bloodstream infections among hospitalized patients, with a crude mortality rate of 30-81% [4]. Such infections in most cases are characterized by formation of complex, structured microbial communities called biofilms [3]. Fungal biofilm formation is a complex and diverse phenomenon. Formation and expression of biofilms by other yeast pathogens such as *Trichosporon* proceeds through phases and is often associated with increased antifungal resistance due to upregulated drug efflux and other factors [4]. Disseminated trichosporonosis is an uncommon but increasingly reported and frequently a fatal fungal infection in immunocompromised patients. Clinically, trichosporonosis presents with fever, pulmonary infiltrates, azotemia, renal dysfunction, and skin lesions. *Trichosporon* spp. was also isolated from sepsis in immature infants and summer-type hypersensitivity pneumonitis in Japan [2]. Formation of biofilms should preferably be assessed *in vitro* before or during therapy for optimum cure. Test-tube method (TTM) is a good method of biofilm detection *in vitro* but is less sensitive than tissue culture plate method [5]. Microtiter plates for tissue culture method of biofilm assay can be very costly also [6]. There are some drawbacks in the TTM; it has a high degree of subjective variability and is unable to detect moderate to weak biofilm producers; also, petri dishes are widely available [7,8]. Researchers should get better option for detection, assessment of biofilm formation in petri dish method (PDM). A significant proportion of human infections involve biofilms [9]. Microbial biofilms develop when organisms adhere to a surface and produce extracellular polymers [10]. Organisms in biofilms behave differently from freely suspended microbes and have been shown to be relatively refractory to medical therapy [10-12]. Therefore, biofilm-associated infections of retained devices may recur after cessation of antibiotic therapy and hence may necessitate device removal. The formation of bacterial biofilms around devices has been comprehensively investigated [13], but until recently, less focus has been placed on the formation of fungal biofilms. *Trichosporon s*pecies are emerging as an important nosocomial pathogen, and an implanted

device with a detectable biofilm is frequently associated with *Trichosporon* infection [12]. The evidence linking *Trichosporon* biofilms to device-related infections is growing as more standardized methods for evaluating *Trichosporon* biofilms *in vitro* are emerging. Microorganisms growing in a biofilm are intrinsically more resistant to antimicrobial agents than planktonic cells. High antimicrobial concentrations are required to inactivate organisms growing in a biofilm [14]. Biofilms are associated with many medical conditions including indwelling medical devices, dental plaque, and urogenital infections [15]. There are various methods to detect biofilm production. These include the TTM; we postulate the PDM. We tested biofilms in *Trichosporon* spp. by two different methods, which could be used in a routine clinical laboratory, for determining their ability to form biofilm.

## MATERIALS AND METHODS

### Type of Study

This was a laboratory-based observational study, which was carried out in the Department of Microbiology of the institute as a part of summer training cum dissertation.

### Duration of Study

The study was carried out in 3 months starting from July 2016 to September 2016.

### Methods

Ten isolates of *Trichosporon* were randomly chosen from among the yeast isolates grown and retrieved from samples such as urine, blood, and pus in the laboratory of the department. They were then identified by conventional methods such as germ tube test, microscopic morphology of arthroconidia by Dalmau technique on rice extract agar (REA), and sugar fermentation tests.

### Conventional Methods for Yeast Identification

#### *Germ tube test*

The yeast isolates were inoculated in 0.5 ml of pooled fresh human serum and incubated in a water bath at 37°C for 2 h. A drop of suspension (40 µl) was placed on a clean glass slide and examined under microscope (×10 and ×40) for germ tube formation. Germ tube positive is a *Trichosporon.*

#### *Morphology on REA (Dalmau Technique)*

Light inoculum of the yeast isolates was partially streaked into half the thickness of REA (Rice powdered: 0.04 g; agar: 1.5 g; and deionized water: 100 ml), prepared by autoclaving at 121°C for 15 min) media making 4-5 parallel lines of approximately 2-2.5 cm long and 0.5-0.8 cm apart. A flame-sterilized coverslip was placed over it and incubated at 22°C for 3-5 days. After incubation, the colony was observed under the microscope (×10 and ×40 objectives) for typical morphological features such as yeast and arthroconidia. Fungi having budding yeasts and arthroconidia in chains were noted as *Trichosporon* spp.

#### *Sugar fermentation*

Nutrient broth with glucose, maltose, sucrose, and lactose, each in concentration of 2% (weight/volume) with phenol red indicator (0.1% w/v) with appropriate color on cotton plug for differentiation, was prepared, autoclaved, and dispensed (2 ml each) in sterile test tube. One loopful of yeast isolate was inoculated in 2 ml of the liquid medium in test tubes and incubated at 37°C overnight with known strains of *Trichosporon spp.* as controls. A yellow coloration was indicative of positive fermentation reaction.

#### *TTM versus PDM*

Peptone water with 1% (weight/volume) glucose was prepared and autoclaved at 110°C at 10 lbs/in$^2$ pressure. In 3 ml of this media, each in 2 glass test tube, 0.5 McFarland turbidity (standard turbidity) of suspension of each isolate was made. One tube was incubated at 37°C overnight as such and contents of another were dispensed in polystyrene, disposable, sterile, and 90 mm petri dish (Tarsons Inc.). Then, the petri dishes were incubated at 37°C overnight. Next day, liquid contents of both test tube and petri dish were drained off, and test tube and petri dishes were both washed thrice with sterile 0.9% normal saline. After that, 3 ml of 0.5% aqueous safranine was poured in both test tube and petri dish and kept for 1 min. Following this, safranine was drained from both of them. Again, they were washed thrice with 0.9% normal saline. After that, both tube and petri dish were kept inverted for drying. Test tube was observed by naked eye for biofilm formation, and petri dish was observed by naked eye and also microscopically at ×10 and ×40 microscope objectives by focusing it. All tests were done thrice with each isolate.

## RESULTS

PDM is an equally good method to detect biofilm formation as compared to TTM as found in the study. In PDM, biofilms were always found in clusters of many yeast cells. Arthroconidia were not visible. Furthermore, we were able to grade biofilm formation microscopically as 1+ and 2+ visually in PDM. Results have been shown in Table 1. Macroscopic view of biofilm by PDM is shown in Figure 1.

From above experiment 10 isolates of *Trichosporon* spp., we are discussing below the results. TTM was taken as gold standard here.

The sensitivity of PDM was very high, and specificity was also good as compared to TTM. Both sensitivity and specificity were 100%.

**Table 1: Results of TTM and PDM**

| Isolates | TTM | PDM |
|---|---|---|
| *Trichosporon* spp. | Biofilm seen | Seen in clusters |
| *Trichosporon* spp. | Biofilm seen | Seen in clusters |
| *Trichosporon* spp. | Biofilm seen | Clusters formed |
| *Trichosporon* spp. | Biofilm seen (lightly) | Clusters formed |
| *Trichosporon* spp. | Biofilm seen | Clusters formed |
| *Trichosporon* spp. | Biofilm seen | Clusters formed with chains |
| *Trichosporon* spp. | Biofilm seen (lightly) | Clusters formed |
| *Trichosporon* spp. | Biofilm seen | Clusters formed |
| *Trichosporon* spp. | Biofilm seen | Clusters formed |
| *Trichosporon* spp. | Biofilm seen | Clusters formed |

**TTM: Test tube method, PDM: Polystyrene petri dish method**

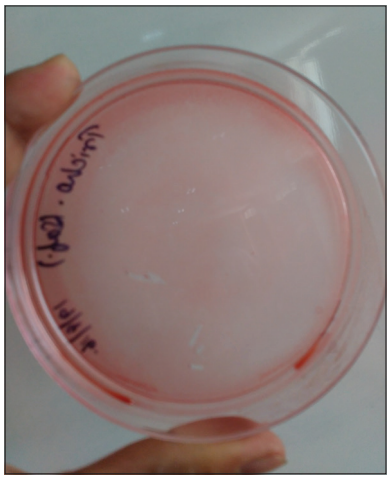

**Figure 1: Macroscopic view of biofilm by petri dish method**

## DISCUSSION

Biofilms are characterized by structural heterogeneity, genetic diversity, complex community interactions, and an extracellular matrix of polymeric substance [16]. Non-albicans *Candida* biofilms are especially widespread and have been observed in most medical devices, such as stents, shunts, implants, endotracheal tubes, pacemakers, and various types of catheters [17-19]. Various methods have been employed for the detection of biofilms. Newer techniques such as DNA extraction and quantification and quantification polymerase chain reaction evaluated of late by other authors, which is time consuming and expensive [20,21]. Compared to these newer techniques, PDM is least expensive. Microscopy strongly suggests that extracellular material is predominantly composed of cell wall such as polysaccharides containing mannose and glucose residues, based on staining with dyes that specifically bind these carbohydrates. *Trichosporon* spp. is one of the emerging mycoses in neutropenic patients, usually in the setting of an hematological malignancy. *Trichosporon asahii* causes white piedra, a superficial infection of hair shafts mainly restricted to tropical regions and less commonly onychomycosis in immunocompetent humans [2]. In immunodeficient hosts, it has been isolated from blood, skin, and viscera causing various localized or disseminated deep infections [22].

In our study, we evaluated ten *Trichosporon* spp. isolates grown and retrieved normally from samples such as urine, blood, and pus put up for culture, thrice each. Biofilm was better observed in PDM compared to TTM. Fungal biofilm formation is a complex phenomenon distinct from simple adhesion. It is best studied using pathogenic species grown on relevant bioprosthetic devices and catheters, and the risk of biofilm development on catheters by yeasts such as *Candida* spp. has been estimated to be up to 30% depending on the location of the catheter [23]. Fungal infections caused by yeasts are an emerging problem in health care as advances in modern medicine prolong the lives of severely ill patients [24]. Increasing drug resistance associated with fungal biofilms is likely multifactorial and among other mechanisms may be due to: (a) high cellular density within the yeast biofilm, (b) the shielding effect of the biofilm exopolymeric matrix, (c) differential expression of genes linked to resistance, including upregulated drug efflux pumps, and (d) the presence of a subpopulation of "persister" or slowly growing cells [23]. There are different methods of studying biofilms *in vitro*, of which microtiter plate or tissue culture method is a good method [25]. However, such expensive techniques are not commonly available for use in routine and peripheral clinical microbiology laboratories. The present study, therefore, evaluated two simple and cost-effective alternatives methods for the identification of *Trichosporon* spp. TTM can be a good method for this purpose, but it has high degree of subjective variability in reading and cannot detect moderate to weak biofilm producers [7]. Polystyrene petri dishes, used mainly for media dispensing, are cheap and easily and widely available [8]. As far as we know, this is the first reported use of petri dishes for routine biofilm production in *Trichosporon* spp. If this method is successful, it can even be done in bedside, and this will be helpful since treatment can then be modified accordingly. We can even grade degree of biofilm formation in this method (PDM), much like TTM. These newer tests are simple and cost effective that will aid routine yeast identification, so this method can be a simple, yet better option for detecting assessing biofilm formation by the notorious yeast pathogen, *Trichosporon* spp. Polystyrene PDM is equally good for biofilm detection as compared to TTM. Furthermore, we were able to grade biofilm formation microscopically as 1+ and 2+ visually in PDM. Thus, gradation of biofilm formation can be done. Furthermore, we can study the effect of methylene blue on biofilms to see metabolic activity of the biofilm cells. In addition, the effect of antifungal drugs can be assessed by incubating with yeasts in this biofilm method.

## CONCLUSION

We can conclude from our study that PDM is a quantitative and reliable method to detect biofilm forming microorganisms. PDM can be recommended as a general screening method for detection of biofilm producing *Trichosporon* in laboratories.

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