Open Access Journal

Indian Journal of Medical Research and Pharmaceutical Sciences

September 2016; 3(9) ISSN: ISSN: 2349-5340

DOI: 10.5281/zenodo.61779 Impact Factor: 3.052

EVALUATION OF POLYSTYRENE PETRI DISH- BASED METHOD FOR ASSESSING BIOFILM FORMATION IN VITRO BY *C.GLABRATA* AND ITS COMPARISON WITH TEST-TUBE METHOD

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Keywords:

Candida glabrata , Peptone water with dextrose, Safranine, Normal saline, Polystyrene petri dish, Test tube.

Abstract

Introduction:- Biofilms formation plays an important role in fungal pathogenesis . Biofilms have been considered a virulence factor contributing to the Candidal infection. Therefore, a reliable method for their diagnosis is necessary. Materials and methods: In this study, biofilm formation of 10 isolates of Candida glabrata by Test tube method and polystyrene petri dish method was compared. Results: Petri dish method was found comparable with test tube method for studying biofim formation with better sensitivity but poorer specificity. Conclusion:- Petri dish method can be safely used to find out pattern of biofilm formation by Candida glabrata.

Introduction

The growth form of microorganisms that is associated with a surface is called a biofilm. The human microbiota plays a role in human metabolism and in understanding the pathogenesis and the optimized therapy for many diseases(1). Fungi being eukaryotic cells and more complex than bacteria cause infections that are often difficult to diagnose and treat, and carry unacceptably high mortality rates (2). Candida bloodstream infections (CBSIs) are the fourth most common infections among hospitalized patients , accounting for 30% to 81% of hospital-acquired Boold stream infections (3). They are considered high-morbidity infections [4, 5], with significant hospital costs (6,7), largely due to increased hospital length of stay and costs for antifungal therapy (8). Use of broad spectrum antibiotics, neutropenia, parenteral nutrition, indwelling catheter are risk factors contributing to increased disease burden(9). This is also the suspected mechanism by which C.glabrata forms microbial biofilms on urinary catheters, and less commonly in-dwelling catheters. It also causes problems with dental devices, such as dentures(10). In addition, the cells of a true biofilm produce their own extracellular matrix material and manifest phenotypes that are distinct from the phenotypes of cells growing in suspension (called planktonic cells). However, in their natural ecosystems, most microbes exist as attached communities of cells within an organized biofilm and rarely as planktonic organisms (11). Thus, a biofilm is defined as a surface associated and highly structured community of microorganisms that are enclosed within a protective extracellular matrix. Microbial biofilms can not only form in nature but also inside a host, and in recent years there has been an increased appreciation of the role that microbial biofilms play an important in human medicine: it is now estimated that about 65% of all human infections have a biofilm etiology (12). Formation and expression of biofilms by yeast pathogens like *Candida glabrata* proceeds through phases, and is often associated with increased antifungal resistance due to upregulated drug efflux and other factors(13). . Fully mature *Candida* biofilms have a mixture of morphological forms and consist of a dense network of yeasts. The formation and structure of *Candida* biofilms is influenced by the nature of the contact surface, environmental factors, *Candida* morphogenesis, and the *Candida* species involved.

Formation of biofilms, therefore should preferably be assessed in vitro before or during therapy for optimum cure. Test tube method is a good method of biofilm detection in vitro but is less sensitive than Tissue culture plate method(14). Microtitre plates for Tissue culture method of biofilm assay can be very costly also(15). There are some drawbacks in the test tube method ; it has a high degree of subjective variability, is unable to detect moderate to weak biofilm producers(16) as compared to polystyrene petri dish method which is a cheap, easily and widely available(17) and simple method . We get better option for detection, assessment of biofilm formation. A significant proportion of human infections involve biofilms(18). Microbial biofilms develop when organisms adhere to a surface and produce extracellular polymers that provide a structural matrix and facilitate further adhesion (11). Organisms in biofilms behave differently from freely suspended microbes and have been shown to be relatively refractory to medical therapy (11, 19, 20). 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 (21), but until recently, less focus has been placed on the formation of fungal biofilms. *Candida* species are emerging as an important nosocomial pathogen, and an implanted device with a detectable biofilm is frequently associated with *Candida* infection (20). The evidence linking *Candida* biofilms to device-related infections is growing as more standardized methods for evaluating *Candida* biofilms in vitro are emerging. Here, we review the role of biofilm production in the pathogenicity of *Candida*-related device infection and the antifungal drug susceptibility of *Candida* biofilms.

Materials and methods

Type of Study

This was a laboratory-based observational study, which was carried out in the Department of Microbiology , All India Institute of Medical Sciences, Patna , 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 *Candida glabrata* was randomly chosen from among the yeast isolates grown and retrieved from samples like urine, blood, and pus in the laboratory of the department. They were then identified by conventional methods like germ tube test, microscopic morphology by Dalmau technique [on Rice extract agar], growth at 44 ℃ 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 ℃ for 2 hours. After incubation, a drop of suspension (40 microlitre) was placed on a clean glass slide and mounted with a flame-sterilised glass cover slip and examined under microscope (10x and 40x) for germ tube formation.

Morphology on Rice Extract Agar (Dalmau Technique) :-

Light inoculum of the yeast isolates was partially streaked into half the thickness of Rice Extract Agar (REA) (Rice, powdered, 0.04 g; Agar agar 1.5 g; Deionized water 100 mL, Prepared by autoclaving at 121 deg℃ for 15 mins.) media making 4-5 parallel lines of approximately 2 to 2.5 cm long and 0.5 to 0.8 cm apart. A flame sterilized cover slip was placed over it and incubated at 22 ℃ for 3 to 5 days. After incubation, the colony was was observed under the microscope (10X and 40X objectives) for typical morphological features like only yeast.

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 colour marking on cotton plug for differentiation were prepared , autoclaved at 110 deg C for 10 mins and dispensed (2 ml each) in sterile test tubes . 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 *Candida spp.* as controls. A yellow colouration was indicative of positive fermentation reaction.

Test Tube Method VS Petri dish method :-

Peptone water with 1% (weight/volume) glucose was prepared and autoclaved at 110 ℃ at 10 Ibs/in^2 pressure. In 3ml of this media each in 2 glass test tube , 0.5 Mc Farland turbidity (standard turbidity) of suspension of each isolates was made. One tube was incubated at 37 ℃ overnight as such and contents of another was dispensed in polystyrene disposable, sterile , 90 mm petri dish(Tarsons Inc.) . Then the petri dishes were incubated at 37 ℃ overnight . Next day , Liquid contents of both test tube and petri dish was drained off and test tube and petri dish 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 minute. 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 10X and 40X microscope objective.

Results

Results have been shown in Table 1

Table 1:-Results of Test tube method and Petri dish method

Serial no.	**ISOLATES**	**TEST TUBE METHOD**	**PETRI DISH METHOD**
1	*C. glabrata*	Biofilm seen	Budding, uniform layer
2	C. *glabrata*	Biofilm seen	Budding yeasts
3	C. *glabrata*	Biofilm seen	Budding yeasts
4	C. *glabrata*	Biofilm seen	Uniform layer
5	C. *glabrata*	Biofilm seen	Uniform layer
6	C. *glabrata*	Biofilm not seen	Uniform, few in clusters
7	C. *glabrata*	Biofilm not seen	Clusters formed
8	C. *glabrata*	Biofilm seen	Uniform layer
9	C. *glabrata*	Biofilm not seen	Uniform layer
10	C. *glabrata*	Biofilm seen	Uniform layer

Thus the Petri dish method was equally good in detecting biofilms as compared to test tube method.

Discussion

Fungal infections caused by yeasts are an emerging problem in health care as advances in modern medicine prolong the lives of severely ill patients(22)Device-related Candida infections are relatively refractory to medical therapy [23]. This is due to the formation of biofilms. 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) Presence of a subpopulation of “persister” or slowly growing cells(24). Certain Candida species in the presence of glucose-containing fluids or lipid emulsion produces biofilm, potentially explaining the increased proportion of CBSIs among patients receiving parenteral nutrition (25- 26). Certain risk factors are associated with biofilm forming CBSI like diabetes, neutropenia and prolonged antibiotics . Diabetes mellitus has previously been reported to be a general risk factor for *Candida* infections[23]. Yet, glucose is thought to serve as the carbohydrate energy source required by *Candida* for biofilm formation [27], perhaps necessary to produce the polysaccharide matrix (27), in which organized communities of yeast are enclosed (20). *C.glabrata* biofilms have a highly heterogenous architecture in terms of distribution of fungal cells and extracellular material(28). Like their bacterial counterparts biofilm grown *C.glabrata* cells are highly resistant to antimicrobials. Although drug resistance has been shown in *C.glabrata* (29,30)and bacterial biofilms (24,22) this is the first report correlating the emergence of antifungal resistance with the development of biofilms. Developing *C.glabrata* biofilms are associated with an increasing presence of extracellular material. It is unclear if the increase in drug resistance in *C.glabrta* biofilms is due to production of extracellular material or due to genetic and biochemical alterations in fungal cells(24,22). In our study, we evaluated 10 *Candida glabrata* isolates grown and retrieved normally from samples like urine, blood, and pus put up for culture. Biofilm better seen in petri dish method compared to Test tube method. Fungal biofilm formation is a complex phenomenon distinct from adhesion. It is best studied using pathogenic species grown on relevant bioprosthetic devices and catheters, and the risk of biofilm development on catheters by *candida spp.*has been estimated to be up to 30% depending on the location of the catheter(31). There are different methods of studying biofilms in vitro, of which microtiter plate or tissue culture method is a good method(22). Also 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 *candida glabrata*. Test tube method 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(32). Polystyrene petri dishes, used mainly for media dispensing, are cheap and easily and widely available, strong biofilm producers(17).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 or petri dish method), much like test tube method. 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 neotorious yeast pathogens. Polystyrene petri dish method is equally good for biofilm detection as compared to test tube method. Also we were able to grade biofilm formation microscopically as 1+, 2+ etc. Thus gradation of biofilm formation can be done. Also, we can study the effect of methylene blue on biofilms to see metabolic activity of the biofilm cells. Additionally, effect of antifungal drugs can be assessed by incubating with yeasts in this biofilm method.

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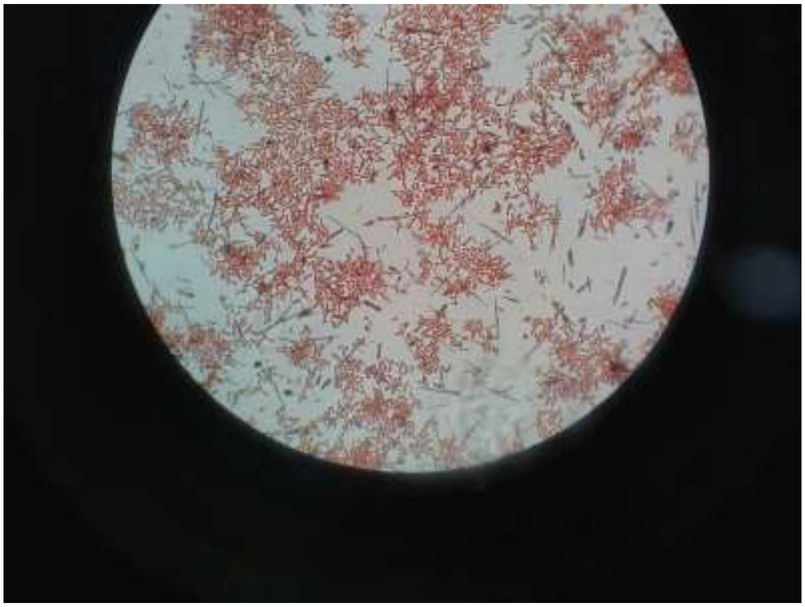

c glabrata biofilm in petri dish

c glabrata biofilm in test tube