Original Article

DOI: 10.7860/JCDR/2014/10445.5147

Microbiology Section

Comparison of Various Methods Used to Detect Biofilm Production of *Candida* Species

RAHUL P. DHALE[1], M. V. GHORPADE[2], C. A. DHARMADHIKARI[3]

ABSTRACT

Purpose: The biofilm of organisms can be considered as a virulence factor because of the resistance towards antimicrobial agents. Formation of *Candida* biofilms are observed in blood, mucosal surface and most medical devices i.e. nonliving objects in patient's body. The study was undertaken to conclude the most sensitive and specific test for detection of biofilm formation.

Materials and Methods: Quantitative measurement of biofilm formation was assessed by three methods- 1) XTT reductionassay, 2) Percentage Transmission (%T) and 3) Crystal Violet assay in microtitre plate for 425 *Candida* isolates.

Results: Out of 425 *Candida* strains, XTT reduction assay gave 72 Biofilm positive strains accounting for 16.94 %. The percentage transmission (% T) method gave 56 strains Biofilm positive (13.17 %) and Crystal violet assay gave 50 positive strains (11.76 %). Compared to Percentage Transmission (% T) and Crystal violet method, XTT reduction assay gave maximum percentage of Biofilm positivity.

Conclusion: In comparison of 3 methods used for detection of biofilm formation, XTT reduction assay was the most sensitive and specific method.

Keywords: Biofilm, XTT reduction assay

INTRODUCTION

Formation of a Biofilm begins with the attachment of free-floating microorganisms to a surface. Fundamental of Biofilm formation is coordination and communication within microbial cells via signaling [through release of acyhomoserine lactone (AHL)] cell to cell called Quorum sensing [1]. *Candida albicans* remains the fungal species most commonly associated with biofilm formation. Increase in *Candida* infections in the last decades has almost paralleled the increase and wide spread use of broad range of medical implant devices [2-4]. There are various methods used to detect biofilm formation. These methods vary widely as to their time and cost requirements like Congo red agar method, CFU, determination of dry weight, scanning electron microscope (SEM) etc. In this study we have selected 3 methods in which 2 methods are crystal violet assay and % Transmission which are reliable, low cost effective and sensitive. And third method is XTT reduction assay which is modest costly but very sensitive, most reproducible, accurate and efficient method widely used for *Candida* biofilm detection [3].

MATERIALS AND METHODS

A total number of 425 cases, clinically diagnosed as Cellulites, Septicemia, Ketoacidosis, Pneumonia, Respiratory infections, Skin infections, Abscess, Diabetes, RVD (Retro Viral Diseases), Renal failure, Heart diseases, UTI (Urinary Tract Infections), Meningitis, Oral lesions, Dental caries etc. were studied. Samples were collected by conventional method from Krishna Hospital Karad, Private Medical Laboratories & hospitals, in an around Kolhapur, Sangli (MS) and from Belgaum (KS). Duration of study was September 2008 to January 2013.

Candida species were isolated on Sabouraud's dextrose agar. Speciation of *Candida* was done by Dalmau plate technique on corn meal agar. Also, sugar assimilation and fermentation tests were performed by using 2% concentration of glucose, maltose, sucrose, lactose, galactose, and trehalose were used for test in which Bromothymol blue indicator was used [5].

Methods used for biofilm production

1) Percentage transmission (% T) or percent transmittance (% T):- Biofilm production was detected by measuring optical density (% T) in microtitre plate with ELISA reader [4,6].

Growth from Sabouraud's agar (SDA) were taken in 5 ml of sterile 0.85% saline to a density of a 0.5 McFarland's nephalometer standard tube no. 3 was matched with growth of *Candida* in testtube which is approximately 10^7 cells/ml. Followed by a 1:20 dilution in Sabouraud's broth with a final concentration of 8% glucose. Then 100 µl of suspension was incubated at 37°C over night (with or without 75 rpm) in commercially available pre-sterilized, polystyrene, flat-bottomed 96-well microtitre plate for biofilm production. The microtitre plate was washed with distilled water in washer (Lablife eW 2007 Microplate ELISA washer, DIAGNOVA) and 200 µl distilled water was added in well and reading was taken in microtitre plate reader (Lablife ER 2007 Microplate washer, DIAGNOVA) at 405 nm and Percent Transmittance (% T) which gave high Optical density was considered as positive for biofilm production.

2) Crystal violet assay:- By above method (%T), after washing with distilled water, microtitre plate was stained with 110 µl of 0.4% aqueous crystal violet solution for 45 min. Afterwards, each well was washed four times with 350 µl of sterile distilled water and immediately destained with 200 µl of 95% ethanol. After 45 min of destaining, 100 µl of destaining solution was transferred to a new well and the destaining solution was measured with ELISA reader (Lablife ER 2007 Microplate washer, DIAGNOVA) at 595 nm [3,7].

3) XTT reduction assay: - It has been used as a routine tool for the quantification of *Candida* biofilms [3,7-11]. {XTT: (2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide)}

By above Percentage transmission (% T) method,100 µl of 10^7 cells /ml (McFarland's nephalometer standard tube no. 3) in Sabouraud's broth with 8% glucose, incubated at 37°C for overnight (with or without 75 rpm) in 96 welled microtitre plate [9].

Sr. No.	Method	Biofilm Positive isolates -Percentage (%)
1	XTT reduction assay	72/425 (16.94 %)
2	Percentage Transmission (% T)	56/425 (13.17 %)
3	Crystal Violet assay	50/425 (11.76 %)

[Table/Fig-1]: Comparison of various methods used to detect biofilm production of 425 *Candida* species

After the adhesion phase, the cell suspensions were gently aspirated and each well was washed twice with Phosphate Buffered Saline (PBS)[5] to remove any remaining planktonic cells, taking care not to disturb the adhered cells. In order to allow the growth of biofilm (Biofilm phase), 200 µL of freshly prepared Yeast Nitrogen Broth, YNB [5] with supplemented with glucose was added to each well [9]. The plates were incubated for 24 h at 37ºC (at 75 rpm in an orbital shaker). After 24 h of incubation XTT reduction assay was performed.

To prepare the standard XTT (Himedia laboratories pvt. Ltd. Mumbai) formulation, XTT was dissolved in sterile PBS at a final concentration of 1 mg/mL [9]. For every assay freshly prepared solution was used. Menadione solution (0.4 mM; Sigma-Aldrich Corp.) was prepared immediately before each assay [9]. For each assay, XTT solution was mixed with menadione solution at a volume ratio of 20:1. The biofilms were washed twice with 200 µL of PBS to remove adherent cells. Next, 158 µL of PBS with 40 µL of XTT and 2 µL of menadione were transferred to each well of 96-well plates. The plates were covered with polythene foil and incubated in dark at 37ºC for 3 h.

Thereafter, 100 µL of the solution was transferred to each well of new 96-well plates. The colorimetric changes were measured at 492 nm using a microtiter plate ELISA reader (Lablife ER 2007 Microplate washer, DIAGNOVA) [3,7-12].

OBSERVATIONS AND RESULTS

There was no signficant diffrence in proportion of Biofilm positive cases detected by XTT reduction assay method and percentag transmission (%T) method (χ^2=2.355, p=0.1249). Similarly there was no significant difference in the proportion of Biofilm positive cases detected by %T method and Crystal violet assay method (χ^2=0.3880, p=0.5333). However, proportion of Biofilm positive cases detected by XTT method was significatntly more than proportion of Biofilm positive cases detected by Crystal violet assay method (χ^2=4.632, p=0.0314).

DISCUSSION

Biofilms are characterised by structural heterogenicity, genetic diversity, complex community interactions, and an extra cellular matrix of polymeric substance [7]. Predisposing factors for production of biofilm in vivo are Skin and mucosal barriers, pathogen induced immunity, blood stream infections and adherence. But not only *Candida albicans* but, also 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 [2,4,13].

There are various methods have been employed for the detection of biofilms. In the present study, the most commonly used method by different authors was XTT reduction assay, which gave 72/425 *Candida* biofilm positive strains, accounting for 16.94%. These 72 *Candida* includes albicans as well non-albicans strains. The percentage transmission (%T) method gave 56/425 strains biofilm positive (13.17%) and Crystal violet assay gave 50/425 positive strains (11.76 %).

All the 56/72 (77.77%) and 50/72 (69.44%) strains detected positive for biofilm formation by Percentage transmission (%T) and Crystal violet assay were the same strains detected by XTT reduction assay [Table/Fig-1].

Girishkumar CP et al., [6] studied biofilm production only by percentage transmission (%T), they found that C albicans gave 42.9 % while non-*Candida albicans Candida* gave 93.1% biofilm positivity from blood stream isolates.

In the present study, *Candida* biofilm positive strains showed 16.94 % by XTT reduction assay, 13.17 % by percentage transmission (% T) method and 11.76 % by Crystal violet assay. Shin JH et al., [4] showed 39.00% (%T) while study of Mohamed SA [13] showed 54.00% (Crystal violet assay) *Candida* biofilm positivity. Other authors have characterized *Candida* species and have done studies regarding their biofilm producibility by various other methods; hence it is difficult to compare findings of the present study with others.

Newer techniques like DNA extraction and quantification, qPCR etc. evaluated of late by other authors, which is time consuming and expensive [3,14]. Compared to these newer techniques, XTT reduction assay is least expensive [3]. Taff HT et al., [3] and Jin Y et al., [7] found that XTT reduction assay is reliable and efficient method than Crystal violet assay.

In the present study, compared to Percentage transmission (%T) and Crystal violet assay, XTT reduction assay gives maximum percentage of biofilm positivity. Our study showed XTT reduction assay is less time consuming and accurate method for the detection of biofilm production [3,9,11,15].

CONCLUSION

Candida biofilms are observed in most medical devices, such as stents, shunts, implants, endotracheal tubes, pacemakers, and various types of catheters nonliving objects in patient's body as virulence factor. For the detection of biofilm production, we can conclude that, XTT reduction assay is seen to be the most sensitive, most reproducible, accurate, efficient and specific method to detect the biofilm production as compared to Percentage transmission (% T) and Crystal violet assay.

REFERENCES

[1] Kumar A, Prasad R. Biofilms. *Jk Science.* 2006;8(1):14-17.
[2] Biofilms- The new microbial order, [homepage on the internet] Available from: medicalmycology.org/Biofilms.htm.
[3] Taff HT, Nett JE, Andes DR, Comparative analysis of *Candida* biofilm quantitation assays. *Medical Mycology*. *Early Online.* 2011: 1–5.
[4] Shin JH, Kee SJ, Shin MJ, Kim S H, Shin DH, Lee SK, et al. Biofilm Production by Isolates of *Candida* Species Recovered from Nonneutropenic Patients: Comparison of Bloodstream Isolates with Isolates from Other Sources. *J Clin Microbiol.* 2002;40(4):1244–48.
[5] Cruickshank's Medical Microbiology, Churchill Livingstone; 12th Edition: Apr 1975 (PBS- page 92).
[6] Girishkumar CP, Menon T. Biofilm production by clinical isolates of *Candida* species. *Medical Mycology.* 2006;44:99-101.
[7] Jin Y, Yip HK, Samaranayake YH, Yau JY, Samaranayake LP. Biofilm-Forming Ability of *Candida albicans* Is Unlikely to Contribute to High Levels of Oral Yeast Carriage in Cases of Human Immunodeficiency Virus Infection. *Journal of Clinical Microbiology.* 2003;41(7):2961–67.
[8] Tobudic S, Kratzer C, Lassnigg A, Graninger W, Presterl E. In vitro activity of antifungal combinations against *Candida albicans* Biofilms. *J Antimicrob Chemother*. 2010;65:271–74.
[9] da SILVA WJ, Seneviratne J, Nipuna Parahitiyawa N, Rosa EAR, Samaramayake LP, CURY AADB. Improvement of XTT Assay Performance for Studies Involving *Candida albicans* Biofilms. *Braz Dent J.* 2008;19(4):364-69.
[10] Al-Fattani MA, Douglas LJ. Biofilm matrix of *Candida albicans* and *Candida* tropicalis: chemical composition and role in drug resistance. *J Med Microbiol.* 2006;55:999-1008.
[11] Lal P, Agarwal V, Pruthi P, Pereira BMJ, Kural MR, Pruthi V. Biofilm formation by *Candida albicans* isolated from intrauterine devices. *Indian J. Microbiol.* 2008;48:438–44.
[12] Pruthi V, Al-Janabi A, Pereira BMJ. Characterisation of biofilm formed on intrauterine devices. *Indian Journal of Medical Microbiology.* 2003;21(3):161-65.
[13] Mohamed SA, Al-Ahmadey ZZ. Biofilm Formation and Antifungal Susceptibility of *Candida* Isolates from Various Clinical specimens. *British Microbiology Research Journal.* 2013;3(4): 590-601.

[14] Sherry L, Rajendran R, Lappin DF. Biofilms formed by *Candida albicans* bloodstream isolates display phenotypic and transcriptional heterogeneity that are associated with resistance and pathogenicity. *BMC Microbiology.* 2014;14(182):1-14.

[15] Bitar I, Khalaf RA, Harastani H, Tokajian S. Identification, Typing, Antifungal Resistance Profile, and Biofilm Formation of *Candida albicans* isolates from Lebanese Hospital Patients. *Bio Med Research International.* 2014:1-10.

PARTICULARS OF CONTRIBUTORS:
1. Student, Department of Microbiology, Krishna Institute of Medical Sciences Deemed University, Karad, District Satara, Maharashtra, India.
2. Professor, Department of Microbiology, Krishna Institute of Medical Sciences Deemed University, Karad, District Satara, Maharashtra, India.
3. Professor, Department of Microbiology, Tatyasaheb Kore Dental College and Research Centre, New Pargaon, District Kolhapur, Maharashtra, India.

NAME, ADDRESS, E-MAIL ID OF THE CORRESPONDING AUTHOR:
Dr. Rahul P. Dhale
Student, Department of Microbiology, Krishna Institute of Medical Sciences Deemed University, Karad,
Satara - 415539, Maharashtra, India.
Phone : 09527936360, E-mail : mrrahuld@yahoo.com

FINANCIAL OR OTHER COMPETING INTERESTS: None.

Date of Submission: **Jun 26, 2014**
Date of Peer Review: **Aug 09, 2014**
Date of Acceptance: **Sep 10, 2014**
Date of Publishing: **Nov 20, 2014**