JOURNAL OF CLINICAL AND DIAGNOSTIC RESEARCH

How to cite this article:
BOSE S, KHODKE M , BASAK S , MALLICK S K.DETECTION OF BIOFILM PRODUCING STAPHYLOCOCCI: NEED OF THE HOUR. Journal of Clinical and Diagnostic Research [serial online] 2009 December [cited: 2009 December 7]; 3:1915-1920.

Available from
http://www.jcdr.net/back_issues.asp?issn=0973-709x&year=2009&month= December &volume=3&issue=6&page=1915-1920 &id=469

ORIGINAL ARTICLE

Detection Of Biofilm Producing Staphylococci: Need Of The Hour

BOSE S *, KHODKE M **, BASAK S *, MALLICK S K ****,**

ABSTRACT

Biofilms are group of microorganisms encased in an exopolymeric coat. They have been associated with a variety of persistent infections that respond poorly to conventional antibiotics. In this study detection of biofilm productions by *Staphylococcus spp*. was done by using Congo red agar (CRA) methods, tube methods (TM) and tissue culture plate (TCP) methods. Out of 179 *Staphylococcus spp.,* 111 were *S.epidermidis* and 68 were *S.aureus*. 44.69% of *S.epidermidis* and 32.96% *S.aureus* were slime producers. 97 isolates were detected as slime producer by TCP method, 76 by TN and 11 by CRA method. High resistances to conventional antibiotics were shown by biofilm producers.

This study summarized the prevalence, antibiotic sensitivity pattern and suitable and reproducible method for detection of biofilm producing *Staphylococci*.

Key Words: Biofilm detection, *Staphylococci*, Congo red agar, Antibiotic resistance

*,(M.D)Professor of Microbiology
**search Assistant, Deptt of Microbiology
***(M.D)Professor of Microbiology
****(M.B.B.S)Tutor, Deptt. of Microbiology
Jawaharlal Nehru Medical College,Wardha(M.S)
Corresponding Author
Dr. S. Bose
Professor of Microbiology
J. N. Medical College
Sawangi (M), Wardha-442004 (M.S.)
E-mail: drseema11ghosh@gmail.com
drseema11ghosh@hotmail.com

Introduction

Biofilms are a group of microorganisms attached to a surface and covered by an exopolysaccharide matrix. Various changes occur during their transition from planktonic to a surface attached community. In response to certain environmental signals, new phenotypic characteristics develop in such bacteria. The first recorded observation concerning biofilm was probably given by Henrici in 1933, who observed that water bacteria are not free floating but grow upon submerged surfaces [1]. Certain surface protein, extracellular proteins, capsular polysaccharides, adhesins (PS/A) and autolysin (encoded by *atIE* gene) are involved in regulation of biofilm production. The *ica* gene codes for intracellular adhesion (ICA) and may also code for TS/A and is required for biofilm production [1],[2],[3].

Biofilms are often site for quorum sensing influencing their formation. Availability of key nutrients, chemotaxis towards surface, motility of bacteria, surface adhesins and presence of surfactants are certain factors which influence biofilm formation.[3],[4]. Using *Bacillus subtilis* from soil, Dr Stanley Wall has shown that a protein called Deg U helps the individual bacteria to decide whether to form a biofilm or not [5]. Biofilm producing *Staphylococci* frequently colonize catheters and medical devices and may cause foreign body related infections. They easily get attached to polymer surfaces.[4],[5],[6] Crampton et al showed that like *S epidermidis*, *S aureus* also has *ica* locus encoding the function of intracellular adhesion and biofilm formation [7]. According to a recent public announcement from National Institute Of Health, more than 60% of all infections are

caused by biofilm [8]. Biofilm organisms have an inherent resistance to antibiotics, disinfectants and germicides. The use of synthetic material for implantation is widely associated with "Implant associated infection" due to biofilm production. In the long run they may be very damaging because of immune complex disease [2],[9],[10].

Aims & Objectives

Keeping all these things in mind, the present study was undertaken to detect the prevalence of biofilm producer and nonproducer *Staphylococci* isolated from clinical materials in our laboratory by three different methods, viz. tissue culture plate (TCP) method, tube method (TM) and Congo red agar (CRA) method and to compare the above mentioned three different methods for biofilm production.

Material And Methods

A total of 179 clinical isolates of *Staphylococci spp.* were isolated from blood, infected devices, skin surface, urine, pus etc. from Indoor patient department (IPD) of a rural hospital with tertiary care in Central India over a period of 1 year. Isolates were identified by Gram staining, catalase and coagulase tests. Reference strains of *Staphylococcus epidermidis* ATCC 35984 (high slime producer),

ATCC35983 (moderate slime producer) and ATCC 12228 (nonslime producer) were also included in this study [11]. Detection of biofilm production of 179 *Staphylococci spp*. was done by following three methods.

1. Tissue culture plate (TCP) method [8],[11]
2. Tube method (TM) [11],[12]
3. Congo red agar (CRA) method [11],[13]

1. Tissue Culture Plate Method

10 ml of Trypticase soy broth with 1% glucose was inoculated with a loopful of test organism from overnight culture on nutrient agar. The broth was incubated at 37^0C for 24 hours. The culture was further diluted 1:100 with fresh medium. 96 wells flat bottom tissue culture plates were filled with 0.2 ml of diluted cultures individually. Only sterile broth was served as blank. Similarly control organisms were also diluted and incubated. All three controls and blanks were put in the tissue culture plates. The culture plates were incubated at 37^0C for 24 hours. After incubation, gentle tapping of the plates was done. The wells were washed with 0.2 ml of phosphate buffer saline (pH 7.2) four times to remove free floating bacteria. Biofilms which remained adherent to the walls and the bottoms of the wells were fixed with 2% sodium acetate and stained with 0.1% crystal violet. Excess stain was washed with deionized water and plates were dried properly. Optical densities (OD) of stained adherent biofilm were obtained with a micro ELISA autoreader at wave length 570 nm. Experiment was performed in triplicate and repeated thrice. Average of OD values of sterile medium were calculated and subtracted from all test values [8],[11].

2. Tube Method

10 ml Trypticase soy broth with 1% glucose was inoculated with a loopful of test organism from overnight culture on nutrient agar individually. Broths were incubated at 37^0C for 24 hours. The cultures were decanted and tubes were washed with phosphate buffer saline (pH 7.3). The tubes were dried and stained with 0.1% crystal violet. Excess stain was washed with deionized water. Tubes were dried in inverted position.

In positive biofilm formation, a visible stained film was seen lining the wall and bottom of the tube. Experiments were done in triplicate for 3 times and read as absent, weak, moderate and strong.[11],[12]

3. Congo Red Method

The medium composed of Brain heart infusion broth (37 gm/l), sucrose (5 gm/l), agar number 1 (10 gm/l) and Congo red dye (0.8 gm/l). Congo red stain was prepared as concentrated aqueous solution and autoclaved at 121^0C for 15 minutes. Then it was added to autoclaved Brain heart infusion agar with sucrose at 55^0C. Plates were inoculated with test organism and incubated at 37^0C for 24 to 48 hours aerobically. Black colonies with a dry crystalline consistency indicated biofilm production [11],[13].

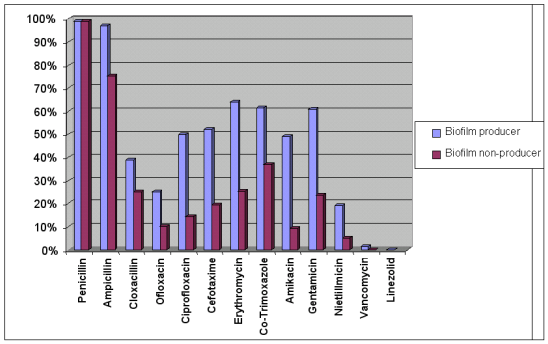
Antibiotic sensitivity test was done on Muller-Hinton agar (MHA) using following antibiotic discs- penicillin (10units), ampicillin(10µg), ofloxacin(5µg), ciprofloxacin (5µg), cefotaxime(30µg), erythromycin(15µg), co-trimoxazole(25µg), amikacin(30µg), gentamicin(10µg), Netillmicin(30µg), linezolid(30µg), vancomycin(30µg), Antibiotics discs were procured from HiMedia Laboratories Pvt. Ltd, India. ATCC *Staphylococcus aureus* 25922 was used as control. Antibiotic sensitivity test was done as per Kirby-bauer disc diffusion method. [14]

Results

[Table/Fig 1] A total of 179 *Staphylococci* were isolated from various clinical materials. Out of 179 *Staphylococcus spp*. 111 *S.epidermidis* and 68 *S.aureus.* Among 111 *S.epidermidis* isolated from different clinical samples, 44.69% were slime producers and 17.32% non-slime producers, whereas among 68 *S. aureus*, 32.96% were slime producers and 5.03% were non-slime producers. Among *S.epidermidis* maximum biofilm producers were from catheter (51 out of 111). Among 51 catheters from which *S.epidermidis* were isolated, 40 were intravenous catheter and 11 were foley's catheters. From two of such catheters adherent slimy growth were seen. Maximum numbers of biofilm producing *S.aureus* were from orthopedic implants (19 out of 68). We found high resistance pattern among biofilm producers in comparison with non-biofilm producers. Two strains of *S. aureus* were intermediate Vancomycin sensitive. Both the strains were biofilm producers [Table/Fig 2].

(Table/Fig 1) Biofilm Production of *Staphylococcal spp.* with regard to source of isolation No. of strains – 179

Source	*S.epidermidis*		*S.aureus*		Total
	Slime +	Slime -	Slime +	Slime -	
Catheters	51	21	8	2	78
Blood	14	6	0	1	21
Orthopaedic implants	13	3	19	2	41
Continuous peritoneal ambulatory dialysis (CAPD)	2	1	1	1	5
Urine	0	0	7	2	9
Throat and nasal swab	0	0	15	0	15
Wound	0	0	9	1	10
Total	80	31	59	9	179
Percentage (%)	44.69	17.32	32.96	5.03	



(Table/Fig 2) Resistance pattern among biofilm producers in comparison with non-biofilm producers

[Table/Fig 3] OD value of stained adherent biofilm was obtained with a microELISA autoreader at wave length 570nm. OD value less than 0.120 was considered as non-biofilm producers, 0.120 -0.240 as moderate biofilm producers and more than 0.240 as strong biofilm producers.

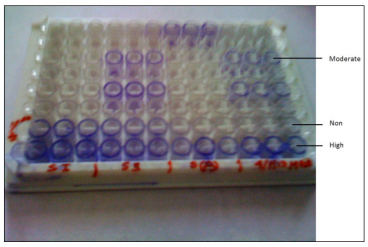
(Table/Fig 3) Classification based on OD values obtained from *Staphylococcus spp.* by TCP method

Mean OD value	Adherence	Biofilm Formation
< 0 .120	Non	Non/weak
0.120 – 0.240	Moderate	Moderate
> 0.240	Strong	Strong

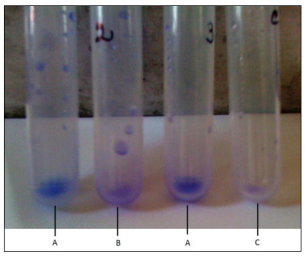
[Table/Fig 4] Among 179 clinical isolates of *Staphylococci*, 15.64% were high biofilm producers by TCP methods,12.30% by TM, and 4.47% by CRA method , whereas 38.55% are moderate biofilm producers by TCP method, 30.16% by TM and 1.68% by CRA method. In TM, 2 were found to be false positive and 23 false negative. In CRA method 3 were false positive and 89 false negative[Table/Fig 5],[Table/Fig 6],[Table/Fig 7].

(Table/Fig 4)Screening of *Staphylococcal* isolates for biofilm formation by TCP, TM and CRA methods

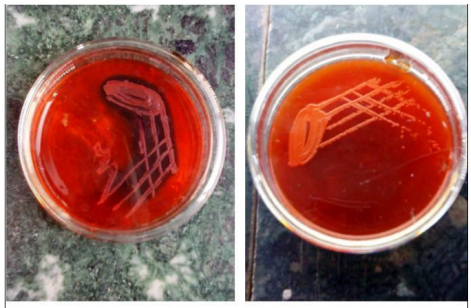
	Biofilm Formation	TCP	TM	CRA
Clinical Isolates n = 179	High	28 (15.64%)	22 (12.30%)	8 (4.47%)
	Moderate	69 (38.55%)	54 (30.16%)	3 (1.68%)
	Weak/Non	82 (45.81%)	103 (57.54%)	168 (93.85%)



(Table/Fig 5) Detection of biofilm producers by TCP method. High, moderate and non slime producers were shown on tissue culture plate.



(Table/Fig 6) Detection of biofilm producers by Tube method. A: High Biofilm producers. B: Moderate biofilm producer. C: Non biofilm producer.



(Table/Fig 6)Detection of biofilm production by Congo Red Agar medium.

A: Colonies of biofilm producer S.epidermidis
B: Colonies of nonbiofilm produce r S.epidermidis

Discussion

Bacterial biofilm has long been considered as a virulence factor contributing to infection associated with various medical devices and causing nosocomial infection [12],[13]

The exact process by which biofilm producing organisms cause disease is poorly understood. However, suggested mechanisms are:

i. Detachment of cells from medical device biofilm causing bloodstream or urinary tract infection.
ii. Endotoxin formation
iii. Resistance to host immune system
iv. Generation of resistance through plasmid exchange [2]

We isolated 179 *Staphylococcal spp.* from clinical samples, namely, blood, pus, urine, dialysis fluid, catheter, nasal swab etc. All isolates were isolated by standard procedure [15] and tested by three in vitro screening tests for biofilm production namely TCP, TM and CRA methods. Out of 179 *Staphylococcal spp.* 111 (62.01%) were *S. epidermidis* and 68 (37.99%) were *S. aureus.* We found that although the formation of biofilm on indwelling medical devices is generally associated with coagulase negative *Staphylococci*, *S. aureus* strains are also capable of production of biofilm (5.03%) which was observed by other workers also. [16],[17]

In this study antibiotic sensitivity pattern of various biofilm producers and non-producer *Staphylococci spp*. Isolated from clinical materials were obtained. The significant and clinically relevant observation was that the high resistance shown by biofilm producers to conventional antibiotics than non-biofilm producers. This observation was supported by other studies also [2],[10]. All strains were sensitive to linezolid and vancomycin except two strains isolated from catheters which were intermediate vancomycin sensitive *Staphylococcus aureus* (VISA). Both were biofilm producers. Glycopeptides may not be optimal antimicrobial agents for the treatment of foreign body associated infection. This may be due to entrapment of vancomycin by the extracellular mucopolysaccharides because of their high molecular weight [10].

We adopted modified TCP method with extended incubation period for 24 hours instead of 18 hours. Trypticase soy broth with 1% glucose medium was used. This method was claimed superior to other methods by various researchers using Trypticase soy broth without glucose and Brain heart infusion broth with sucrose [11].

In TCP method biofilm formation was observed in 97 (54.19%) isolates and non-biofilm producers were 82 (45.81%). This study is similar to the observation made by Mathur et al [11]. In tube test method, 76 (42.46%) isolates were found as biofilm producers whereas 103 (57.54%) were non-biofilm producers. In CRA, 11 (6.15%) strains produced biofilm and 168 (93.85%) were non-biofilm producers. Rate of positivity in CRA method in our study is higher than that of Mathur et al.

For data calculation, OD values obtained for individual strains of *staphylococci spp*.[11] mean OD values < 0.120 was considered non-biofilm producer, 0.120 – 0.240 was moderate and > 0.240 was considered as strong biofilm producers. Modified TCP method was considered as gold standard for this study as various researchers proved this method superior to standard TCP method using Trypticase soy broth without glucose. [8],[11]

Considering modified TCP as gold standard, data from TM and CRA methods were compared. Parameters like sensitivity, specificity, negative predictive value (NPV) and positive predictive value (PPV) were calculated. True biofilm producers were positive by modified TCP, TA and CRA. False positive were biofilm producers by TM and CRA method but not by modified TCP method. False negatives were non-biofilm producers by TM and CRA methods but the same strains were biofilm producers by modified TCP method. True negatives were non-biofilm producers by all the methods. In our study 3 strains gave false positive result and 89 false negative by CRA method. By TM only 2 strains were false positive and 23 false negative considering modified TCP method as gold standard.

Comparative analytical study of TM and CRA methods, with respect to modified TCP method which was considered as gold standard in this study, was as follows: Sensitivity of CRA method was 8.25%; specificity 96.34%; PPV 72.72%; and NPV 47.02%. Sensitivity of TM method was 76.27%; specificity 97.56%; PPV 97.36%; and NPV 77.66%.

Our study shows TCP is the better screening test for biofilm production than CRA and TM. The test is easy to perform and assess both qualitatively and quantitatively. In our study, positivity rate of CRA method was higher than observed by other workers, e.g. Mathur et al. Who has reported 5.26% biofilm producers by CRA method.

There are some highly accurate methods like PCR analysis to detect *ica* genes as virulence marker of staphylococcal infection. Biofilm non-producers are negative for *icaA* and *icaD* and lack the entire *ica ADBC* operon.[13,17] But in a developing country like ours, a low cost method for detection of biofilm is needed which require inexpensive equipment and less technical expertise.

Conclusion

Biofilm can be composed of a single or multiple organisms on various biotic and abiotic surfaces. There is association between biofilm production with persistent infection and antibiotic failure.[19] Hence, in infection caused by biofilm producing *staphylococci*, the differentiation with respect to its biofilm phenotype might help to modify the antibiotic therapy and to prevent infection related to biomedical devices. A suitable and reproducible method is necessary for screening of biofilm producers in any healthcare setup and this TCP method can be recommended.

References

[1] Toole GO, Kaplan HB, Kolter R. Biofilm formation as microbial development. Annual review of Microbiology 2000; 54:49-79.

[2] Donlan RM, Costerton W. Biofilms: Survival mechanisms of clinically relevant Microorganisms. Clinical microbiological review 2002; 15(2): 167-193.

[3] Carol A, Kumamoto, Marcelo DV. Alternative Candida albicans life style: Growth on surfaces. Annual Review of Microbiology 2005; 59:113-133.

[4] Thomas D and Day F. Biofilm formation by plant associated bacteria. Annual review of microbiology 2007; 61:401-422.

[5] Murray EJ, Kiley BT, Stanley-wall RN. A Pivotal role for the response regulator DegU in controlling multicellular behavior. Microbiology 2009; 155: 1-8.

[6] Schwank S, Rajacic Z, Zimmerli W, Blaser J. Impact of bacterial biofilm formation on in vitro and in vivo activities of antibiotics. Antimicrobial agents and chemotherapy 1998; 42(4): 895-898.

[7] Crampton SE, Gerke C, Sehnell NF, Nicols WW, Gotz F. The intracellular adhesion *(ica*) locus present in staphylococcus aureus and is required for biofilm formation. Infection and Immunity 1999; 67(10):5427-5433.

[8] Kim L. Riddle of biofilm resistance. Antimicrobial agents and chemotherapy 2001; 45(4): 999-1007.

[9] Raad I, Darouiche R, Hachem R, Sacilowski M, Bodey GP. Antibiotics and prevention of microbial colonization of catheters. Antimicrobial Agents and Chemotherapy 1995; 39(11): 2397-2400.

[10] Souli M, Giamarellou H. Effects of slime produced by clinical isolates of coagulase negative Staphylococci on activities of various antimicrobial agents. Antimicrobial Agents and Chemotherapy 1998; 42(4): 939-941.

[11] 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 Journal of Medical Microbiology 2006; 24(1):25-29.

[12] Christensen GD, Simpson WA, Bisno AL, Beachey EH. Adherence of slime producing strains of Staphylococcus epidermidis to smooth surfaces. Infection and Immunity 1982; 37(1):318-326.

[13] Arciola CR, Baldassarri L, Montanaro L. Presence of *ica*A and *ica*D genes and slime production in a collection of Staphylococcal strains from catheter associated infections. Journal of clinical microbiology 2001; 39(6):2151-2156.

[14] Bauer AW, Kirby WMM, Sherris JC, Jurek M. Antibiotic susceptibility testing by a standardized single method. American Journal of Clinical Pathology 1966; 45:493-496.

[15] Miles RS, Amyes SGB. Laboratory control of antibicrobiol therapy, in Chapter: 8 Mackie & McCartney's Practical Medical Microbiology14th ed In: JG Collee, AG Fraser, BP Marmion, A Simmons, Editors. Churchill Livingstone: Indian Reprint; 2008. p. 151-178.

[16] Ammendolia MG, Rosa RD, Montanaro LM, Arciola CR, Baldassarri L. Slime production and expression of the slime associated antigens by Staphylococcal clinical isolates. Journal of clinical microbiology 1999; 37(10):3235-3238.

[17] O'Gara JP, Humphreys H. Staphylococcus epidermidis biofilms: importance and implications. Journal of Medical microbiology 2001; 50:582-587.

[18] Ludwicka A, Switalski LM, Ludlin A, Pulvever G, Wadstrom T. Bioluminescent assays for measurement of bacterial attachment to polyethylene. Journal of Microbiological Methods 1988; 26:175-177.

[19] Simon AL, Robertson GT. Bacterial and fungal biofilm infections. Annual review of Medicine 2008. 59:415-428.