Detection of Biofilm Producing *Staphylococci* from Clinical Isolates from Tertiary Care Hospital.

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Abstract:

Biofilm producing organisms are covered with exopolymeric coat and always associated with a variety of persistant infections that respond poorly to conventional antibiotics. In the present study detection of biofilm production by *staphylococci spp* was done by using tissue culture plate (TCP), Congo red agar(CRA) and tube method(TM). Out of total 90 isolates of *staphylococci spp* 52 were *S. epidermidis* and 38 were *S. aureus*. 59% isolates were detected as slime producers by TCP method, 54% by TM and 12% by CRA method. Biofilm producers also shows the high resistance to conventional antibiotics. In this study the attempt has been made to develop a suitable and reproducible method for detection of biofilm producing *Staphylococci.*

Keywords:
Biofilm detection, *Staphylococci*, Congo red agar. Antibiotic resistance.

Introduction:

Biofilms are a group of microorganisms attached to a surface and covered by an exopolysaccharide matrix. 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 (22).

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.(23),(18),(12). Biofilm producing Staphylococci frequently colonize catheters and medical devices and may cause foreign body related infections. They easily get attached to polymer surfaces.(18),(12),(15) Crampton et al showed that like S epidermidis, S aureus also has ica locus encoding the function of intracellular adhesion and biofilm formation (6). 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.

Aims and Objectives:

The present study was undertaken to detect the prevalence of biofilm producer and non producer 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 90 clinical isolates of Staphylococci spp. were isolated from blood, infected devices, skin surface, urine, pus etc. from Indoor patient over a period of 3months. 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 (non slime producer) were also included in this study (10). Detection of biofilm production of 90 Staphylococci spp. was done by following three methods.

1. Tissue culture plate (TCP) method (8),(10)
2. Tube method (TM) (10),(5)
3. Congo red agar (CRA) method (10),(2)

1. Tissue Culture Plate Method

The test organism was grown on nutrient agar platefrom it a loopful is inoculatedin10 ml of Trypticase soy broth with 1% glucose. The broth was incubated at 370C for 24 hours,after incubation the culture was further diluted 1:100 with fresh medium of trypticase soy broth. 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 370C 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. 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),(10).

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 370C 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.(10),(5)

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 1210C for 15 minutes. Then it was added to autoclaved Brain heart infusion agar with sucrose at 550C. Plates were inoculated with test organism and incubated at 370C for 24 to 48 hours aerobically. Black colonies with a dry crystalline consistency indicated biofilm production (13), (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)

Result and discussion:

(Table/Fig 1) A total of 90Staphylococci were isolated from various clinical materials. Out of 90 Staphylococcus spp. 52 S. epidermidis and 38 S. aureus. Among 52 S. epidermidis isolated from different clinical samples, 69.42% were slime producers and 30.56% non-slime producers, From all types of clinical isolates the majority of biofilm producers were isolated from catheters and then from orthopedic implants. Maximum isolates are from catheters which are responsible for slime (31 out of 90).From two of such catheters adherent slimy growth were seen. 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 4) Among 90 clinical isolates of Staphylococci, 31.11% were high biofilm producers by TCP methods, 22.22% by TM, and 4.44% by CRA method , whereas 28.88% are moderate biofilm producers by TCP method, 32.22% by TM and 6.66% by CRA method. Out of 90 isolates88.88% were non or weak slime producers by CRA, 45.55% by TM and 40.00 % by TCP method. (Table/Fig 5), (Table/Fig 6), (Table/Fig 7). The detection of the biofilm production was carried out by taking OD value of stained adherent biofilm with a micro ELISA auto reader at wave length of 570 nm. The range of OD less than 0.120 was considered as non slime producers, between0.120 to 0. 240 were the moderate slime producers and more than 0.240as strong or high biofilm producers.

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 90 Staphylococcal spp. from clinical samples, namely, blood, urine, 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 90Staphylococcal spp.52 (57.77%) were S. epidermidis and 38 (42.22%) were S. aureus. 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. 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 (13).

In TCP method biofilm formation was observed in 54 (60%) isolates and non-biofilm producers were 36 (40%). This study is similar to the observation made by Mathur et al (13). In tube test method, 49 (54.44%) isolates were found as biofilm producers whereas 77 (82.44%) were non-biofilm producers. In CRA, 10(11.11%) strains produced biofilm and 80 (88.88%) 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.(13)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),(13)

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.

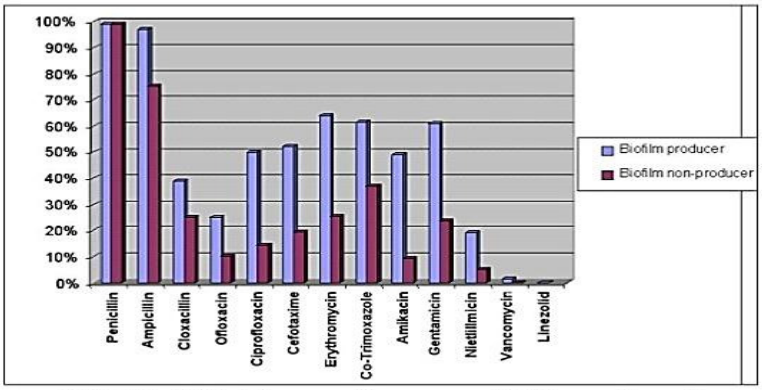
There are some highly accurate methods like PCR analysis to detect ica genes as virulence marker of staphylococcal infection. Biofilm non-producers are negative for icaA and icaD and lack the entire ica ADBC operon.[13,17] But in a developing country like ours, a low cost method for detection of biofilm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

(Table./Fig.1): Biofilm Production of Staphylococci Spp .with respect to Source of isolation No. of Strains 90

Source	**S.epidermidis**		**S.aureus**		**Total**
	Slime(+)	**Slime (-)**	**Slime(+)**	**Slime (-)**	
Cathters	15	4	11	3	33
Blood	6	3	5	3	17
Orthopedic implant	12	3	9	3	27
Wound	2	2	0	2	6
Urine	1	0	0		1
Throat and Nasal swab	2	2	0	2	6
Total	38	14	25	13	90

(Table/Fig4): Screening of Staphylococcal isolates for biofilm formation by TCP,TMand CRA methods.

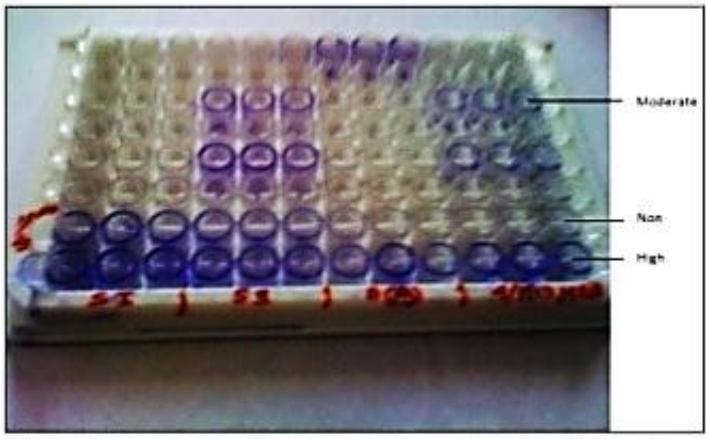
Clinical Isolates N=90	**Biofilm Formation**	**TCP**	**TM**	**CRA**
	High	28 (31.11%)	20 (22.22%)	4 (4.44%)
	Moderate	26 (28.88%)	29 (32.22%)	6 (6.66%)
	Weak/Non	36 (40.00%)	41 (45.55%)	80 (88.88%)



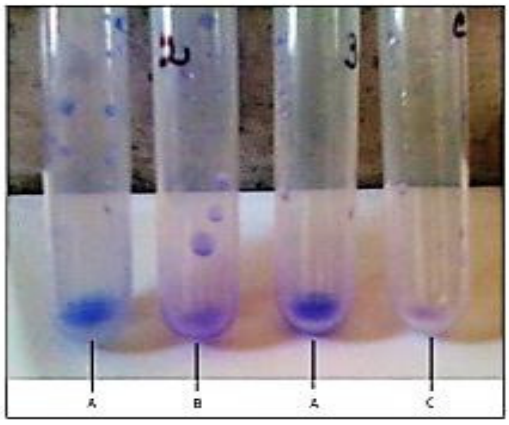
(Table/Fig 2) Resistance pattern among biofilm producers in comparison with non-biofilm producers

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

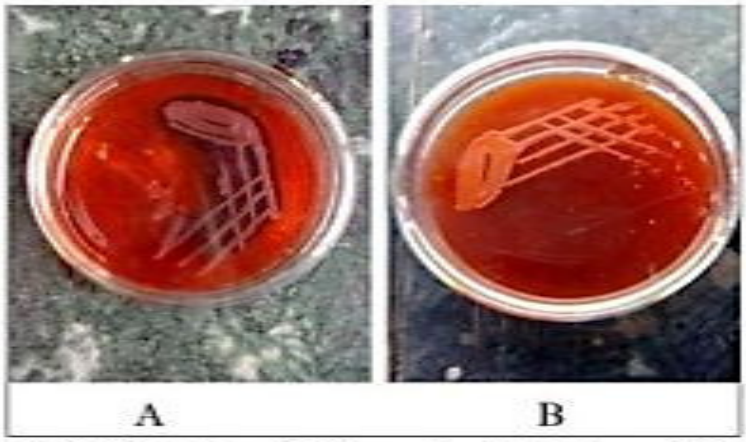
Mean OD value	Adherence	Biofilm Formation
< 0 .120	Non	Non/weak
0.120 – 0.240	Moderate	Moderate
> 0.240	Strong	Strong



(Table/Fig 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

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.(22) 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.

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