

Detection of Biofilm Producing Staphylococci By Three Different Methods and Their Antimicrobial Susceptibility Pattern in Urinary Catheter Tip Isolates

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ABSTRACT

Introduction: 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.

Purpose: To evaluate three different methods for detection of biofilm formation in staphylococci.

Methods: For detection of biofilm formation, 40 clinical isolates of staphylococcus spp. were screened by Congo red agar (CRA) methods, Tube methods (TM) and Tissue culture plate (TCP) methods.

Results: Out of 40 *Staphylococcus* spp., 26 were coagulase negative staphylococci (CNS) and 14 were coagulase positive staphylococci (CPS). 57.6% of CNS and 50% of CPS were slime producers. 22 isolates were detected as slime producer by TCP method, 18 by TM and 12 by CRA method. High resistances to conventional antibiotics were shown by biofilm producers.

Conclusion: The TCP method was found to be most sensitive, accurate and reproducible screening method for detection of

biofilm formation by staphylococci and has advantage of being a quantitative model to study the adherence of staphylococci on bio-medical devices.

Key words: Biofilm Detection, *Staphylococcus*, Tissue Culture Plate (TCP), Congo Red Agar (CRA), Tube Method (TM), Antibiotic Resistance.

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INTRODUCTION

A biofilm is a complex aggregate of microorganisms in which cells adhere to a surface to each other (micro colony). These adherent cells are embedded within a self-produced matrix of extracellular polymeric substances (EPS)/slime, which is made up of proteins and polysaccharides.

Biofilm are universal, occurring in aquatic and industrial water systems as well as large number of environments and medical devices relevant for public health.¹

The first recorded observation concerning biofilm was given by Henrici in 1933; who observed that water bacteria were not free floating, but that they grow on submerged surfaces.²

Certain surface proteins, extracellular proteins, capsular polysaccharides adhesins (PS/A) and autolysin (encoded by *atE* gene) are involved in regulation of biofilm production. The *ica* gene codes for intracellular adhesion (ICA) and may also code for PS/A and is required for biofilm production.^{2,4}

Both the gram positive and gram negative bacteria have the ability to form biofilms. Bacteria commonly involved include *Enterococcus faecalis*, *Staphylococcus aureus*, *Staphylococcus*

epidermidis, *Streptococcus viridans*, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis* and *Pseudomonas aeruginosa*.⁵

Biofilm producing *Staphylococci* frequently colonize catheters and medical devices and may cause foreign body related infections. They easily get attached to polymer surfaces.⁶⁻⁸ Crompton et al showed that like *S. epidermidis*, *S. aureus* also has *ica* locus encoding the function of intracellular adhesion and biofilm formation.⁹ According to a recent public announcement from National Institute Of Health, more than 60% of all infections are caused by biofilm.¹⁰ Biofilm organisms have an inherent resistance to antibiotics, disinfectants and germicides.

In a biofilm, bacteria communicate with one another using chemical signal molecules. This process of chemical communication, called quorum sensing, allows bacteria to monitor the environment for other bacteria and to alter the behavior in responses to changes in a community.¹¹

Availability of key nutrients, chemotaxis towards surface, motility of bacteria, surface adhesins and presence of surfactants are certain factors which influence biofilm formation.

AIMS & OBJECTIVES

The present study was undertaken to detect the prevalence of biofilm producer and nonproducer *Staphylococci* isolated from clinical samples in our laboratory by comparing three different methods, viz. tissue culture plate (TCP) method, tube method (TM) and Congo red agar (CRA) method.

MATERIALS AND METHODS

The present study was conducted in the Department of Microbiology, SMS Medical College and Attached Hospitals, Jaipur (Rajasthan), over a period of one year From October 2011 to September 2012. A total of 40 non-repetitive clinical isolates of *Staphylococci* obtained from urinary catheter tips were included in study. Samples were received from patients admitted in the various wards and intensive care units (ICUs) of the hospital during this period. Detailed relevant history such as age, sex, primary disease and associated predisposing diseases was obtained from patients. All the specimen were inoculated on appropriate culture media like blood agar, Mac Conkey agar and incubated for 24 hour at 37°C. after incubation organism were identified by standard microbiological procedures gram stain appearance, colonial morphology, catalase test, coagulase test.¹² 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.¹³

Detection of biofilm production of 40 *Staphylococci spp.* was done by following three methods.

1. Tissue culture plate (TCP) method.^{10,13}
2. Tube method (TM).^{13,14}
3. Congo red agar (CRA) method.^{13,15}

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°C 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°C 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.^{10,13}

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°C 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.^{13,14}

3. Congo Red Method: The medium composed of Brain heart infusion broth (37 gm/l), sucrose (5gm/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°C for 15 minutes. Then it was added to autoclaved Brain heart infusion agar with sucrose at 55°C. Plates were inoculated with test organism and incubated at 37°C for 24 to 48 hours aerobically. Black colonies with a dry crystalline consistency indicated biofilm production.^{13,15}

Antibiotic sensitivity test was done on Muller-Hinton agar (MHA) using following antibiotic discs- amoxycillin-clavulanic acid (20/10 mg), Clindamycin (2 µg), oxacillin (1µg), ciprofloxacin (5µg), erythromycin (15µg), ticarcillin- Clavulanic acid (75/10µg), gentamicin (10µg), doxycycline (30µg), linezolid (30µg), vancomycin (30µg). Antibiotics discs were procured from HiMedia Laboratories.

ATCC *Staphylococcus aureus* 25922 was used as control. Antibiotic sensitivity test was done as per Kirbybauer disc diffusion method.

Table 1: Biofilm production of Staphylococci.

Source	CPS		CNS		Total
	BP	NBP	BP	NBP	
Urinary Catheter tips	07	07	15	11	40

CPS = Coagulase positive Staphylococci; CNS = Coagulase negative Staphylococci;

BP = Biofilm producer; NBP = Non Biofilm producer

Table 2: Classification of bacterial adherence by TCP Method

Mean OD values	Adherence	Biofilm formation
<0.120	None	None/weak
0.120-0.240	Moderate	Moderate
≥0.240	Strong	High

OBSERVATIONS

A total of 40 *Staphylococci* were isolated from urinary catheter tips. Out of 40 *Staphylococcus spp.* 26 CNS and 14 CPS. Among 26 CNS isolates, 37.5 % were slime producers and 27.5% non-slime producers, whereas among 15 CPS, 17.5% were slime producers and 17.5% were non-slime producers. [Table1]

Out of three methods TCP method detected strong biofilm production in maximum number of isolates 17.5%, whereas detection of strong biofilm production by TM and CRA methods was seen 15% and 7.5% respectively. The TCP method had also

detected more moderate biofilm producing bacteria 37.5% as compared to other methods i.e. 30% and 22.5% by the TM and CRA methods respectively. [Table 3]

Biofilm producers are more resistant to the various antibiotics as compared to the non-biofilm producers. Vancomycin and linezolid were 100% effective both groups. BF isolates were 100% resistant to ticarcillin/clavulanic acid as compared to 88.88% in NBF isolates. All the drugs were more resistant against the BF isolates than NBF isolates but no significant difference was found in the resistance pattern of the between both the groups. [Table 4]

Table 3: Grading of biofilm formation in Urinary Catheter Tips by the three different methods (n=40)

Biofilm formation	TCP %	TM %	CRA %
High	07(17.5%)	06(15%)	03(7.5%)
Moderate	15(37.5%)	12(30%)	09(22.5%)
Weak/None	18(45%)	22(55%)	28(70%)

Table 4: Antibiotic Resistance Pattern (in %) of biofilm forming (BF) and non-biofilm forming (NBF) Staphylococci in Urinary Catheter Tips isolates (n=40)

Antibiotics	Resistance in % of BF* isolates (n=22)		Resistance in % of NBF† isolates (n=18)		P value
	n	%	N	%	
Amoxiclav	18	81.81	10	55.55	0.145
Ciprofloxacin	15	68.18	08	44.44	0.234
Clindamycin	19	86.36	11	61.11	0.142
Erythromycin	20	90.90	12	66.66	0.131
Gentamicin	18	81.81	10	55.55	0.145
Oxacillin	18	81.81	14	77.77	0.937
Vancomycin	0	0	0	0	NA
Doxycycline	16	72.72	14	77.77	1.000
Linezolid	0	0	0	0	NA
Ticarcillin/Clavulanic acid	22	100	16	88.88	0.382

*=Biofilm Forming; †=Non Biofilm Forming; NA=Not Applicable

DISCUSSION

Bacterial biofilm has long been considered as a virulence factor contributing to infection associated with various medical devices and causing nosocomial infection.^{17,18} The different mechanisms, by which biofilm producing organism cause disease are detachment of the cells from medical device biofilm causing bloodstream and urinary tract infection, endotoxin formation, resistance to host immune system and generation of resistance through plasmid exchange.³

In the present study, we isolated 40 strains of Staphylococci from samples of urinary catheter tips, received from different wards and ICUs of SMS Medical College & Attached Hospitals, Jaipur, over a period of one year. Biofilm detection was done by three different methods i.e. Tissue Culture Plate method, Tube method and Congo Red Agar method. We compared the results of different methods, along with determination of antimicrobial susceptibility pattern of the isolates.

Out of 40 staphylococci spp; we isolated 14 (35%) and 26 (65%) CPS and CNS respectively. Similarly CNS is reported as predominant organism in catheter tips by Ammendolia et al.¹⁹

Bose et. al. also reported CNS as predominant isolate i.e. 12% CPS and 88% CNS from indwelling devices.¹

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.^{19,20}

In this study antibiotic sensitivity pattern of various biofilm producers and non-producer *Staphylococci spp.* was studied. 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.^{21,22} All strains were sensitive to linezolid and Vancomycin.^{18,23}

In TCP method biofilm formation was observed in 22 (55%) isolates and non-biofilm producers were 18 (45%). This study is similar to the observation made by Mathur et al and Bose et al.¹¹ In tube test method, 18 (45%) isolates were found as biofilm producers whereas 22 (55%) were non-biofilm producers. In CRA, 12 (30%) strains produced biofilm and 28 (70%) were non-biofilm

producers. Rate of positivity by CRA method in our study is higher than that of Mathur et al. but quite similar to Bose et al.

Comparative analytical study of TM and CRA methods, with respect to TCP method which was considered as gold standard in this study, was as follows: Sensitivity of CRA method was 60%; specificity 81.81%; PPV 50%; and NPV 88%. Sensitivity of TM method was 70%; specificity 88.88%; PPV 81.81%; and NPV 80%. Similar results were observed from other studies also.^{23,24}

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.

CONCLUSION

Bacteria that adhere to implanted medical devices or damaged tissue can become the cause of persistent infection. The increasing use of catheters, artificial implants and antimicrobials as well as high numbers of immunocompromised patients are major causes for concern over biofilm infections. These infections are characterized particularly by high resistance to antimicrobials and formation of persistent foci that may complicate therapy and lead to chronic infections. Therefore, detection of biofilm formation is of high relevance to the clinician and his/her approach to the treatment.

RECOMMENDATIONS

- Use of Tissue Culture Plate (TCP) method for accurate detection of biofilm producers.
- Vancomycin and linezolid as drugs of choice to treat Staphylococcal biofilm formation in suspected patients, as these drugs are effective, relatively safe and can be used in patients of all ages.

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