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Detection of Biofilm Producing Staphylococci And Their Antimicrobial Susceptibility Pattern From Pus Samples in A Tertiary Health Care Centre

KEYWORDS: Biofilm, Staphylococci, Tissue culture plate

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ABSTRACT *Biofilms are microbial communities of the surface-attached cells, embedded in a self-produced extracellular polymeric matrix*

Aim: To detect the biofilm producing Staphylococci from pus samples and compare the different methods used for biofilm detection.

Methods & Results: In the present study, three different methods for detection of biofilm formation were used and their results were compared to find out the most appropriate method for demonstrating biofilm. Out of the 78 isolates, the Tissue Culture Plate (TCP) method detected biofilm in 41 isolates (52.56%), Tube Method (TM) detected biofilm in 32 isolates (41.03%) and Congo Red Agar (CRA) method detected biofilm in 4 isolates (5.13%). The present study showed the TCP method to be most sensitive for the biofilm detection, followed by TM and CRA method.

INTRODUCION

Biofilms are microbial communities of the surface-attached cells, embedded in a self-produced extracellular polymeric matrix.[1] Bacteria often exist as sessile communities in nature called biofilms that develop structures which are both physiologically and morphologically different from free living bacteria. This is mediated by a cell to cell signal mechanism.[2] Biofilm can foster increased resistance to various kinds of environmental stresses as well as antimicrobial tolerance, opportunity of horizontal gene transfer and consortial metabolism. High density of microorganisms within the biofilm provides better opportunity for performing various processes that single cells may not efficiently accomplish, like production of exoenzymes or metabolites which can be effective only above a certain threshold concentration.[3] Biofilms may consist of a single microbial species or multiple microbial species and may form on a range of biotic and abiotic surfaces. Usually mixed-species biofilms are found predominantly in most environments. Single-species biofilms are more commonly encountered in a variety of infections and on surface of medical implants.[4] The ability to form biofilm is seen in both gram positive and gram negative bacteria. Some commonly involved bacteria are *Staphylococcus aureus, Staphylococcus epidermidis, Enterococcus faecalis, Streptococcus viridians, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Proteus mirabilis*.[5]

MATERIALS AND METHOD

The present study was conducted in the Department of Microbiology of National Institute of Medical sciences and Research, Jaipur over a period of one year from June 2014 to May 2015. 78 isolates were obtained from pus samples collected from patients admitted in different wards and intensive care units (ICU's) of the Hospital .Isolates were identified to the species and genus level by standard protocols. The isolates were examined for biofilm formation by three different methods i.e. Tissue culture plate (TCP), Tube Method (TM) and Congo Red Agar (CRA) method. Antimicrobial susceptibility testing was done using Kirby-Bauer disc diffusion method.

Tissue culture plate (TCP)

The TCP assay described by Christensen et al.[6] is most widely used and was considered as standard test for detection of biofilm formation. Isolates from fresh agar plates were inoculated in brain heart infusion (BHI) broth with 2% sucrose dispensed in 2ml amounts in the test tubes, and incubated for 18-24 hours at 37°C in stationary condition. Then the broth with the growth was diluted to 1 in 100 with fresh medium. Individual wells of sterile polystyrene 96 well-flat bottom tissue culture plates were filled with 0.2ml aliquots of the diluted cultures and only broth served as control to check sterility and nonspecific binding of media.

The tissue culture plates were incubated for 24 hours at 37°C. After incubation, the content of each well was gently removed by tapping the plates. The wells were washed four times with 0.2ml of phosphate buffer saline (PBS- pH 7.2) to remove free-floating 'planktonic' bacteria. Biofilms formed by adherent 'sessile' organisms in plate were fixed with sodium acetate (2%) for half an hour and stained with crystal violet (0.1%w/v) for half an hour. Excess stain was rinsed off by thorough washing with de-ionized water and plates were kept for drying. Adherent staphylococcal cells usually formed biofilm on all side wells and were uniformly stained with crystal violet. Optical density (OD) of stained adherent bacteria was determined with a micro ELISA auto reader at wavelength of 570nm (OD_{570nm}). These OD values were considered as an index of bacteria adhering to surface and forming biofilms.

Tube Method (TM)

A qualitative assessment of biofilm formation was determined as previously described by Christensen et al.[7] BHI broth with 2% sucrose (10 ml) was inoculated with loopful of microorganisms from overnight culture plates and incubated for 24 hours at 37°C. The tubes were decanted and washed with PBS (pH 7.3) and dried. Dried tubes were stained with crystal violet (0.1%) for half an hour. Excess stain was removed by washing the tubes with de-ionized water. Tubes were then dried in inverted position and observed for biofilm formation.

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Biofilm formation was considered positive when a visible film lined the wall and bottom of the tube. Tubes were examined and the amount of biofilm formation was scored as 0-absent, 1-weak, 2-moderate or 3-strong.

Congo Red Agar Method (CRA)

This is an alternative method of screening biofilm formation by *Staphylococcus* isolates as described by Freeman et al,[8] which requires the use of a specially prepared solid medium- Brain Heart Infusion broth (BHI) supplemented with 5% sucrose and Congo red. The Congo red agar plates were inoculated with microorganisms from an overnight culture plate and incubated at 37°C for 24-48 hours.

Positive result was indicated by black colonies with a dry crystalline consistency.

RESULTS

A total of 78 isolates were obtained from pus samples collected from patients admitted in different wards and intensive care units (ICU's) of the Hospital of National Institute of Medical Sciences & Research, Jaipur. The bacterial isolates were identified on the basis of standard microbiological procedures like gram staining, colony morphology, catalase test and coagulase test. The isolates were examined for biofilm formation by three different methods i.e. Tissue culture plate (TCP), Tube Method (TM) and Congo Red Agar (CRA) method. Antimicrobial susceptibility testing was done using Kirby-Bauer disc diffusion method. The mean age of patients was 35.5±19.59 years. Males were 51.28% while females were 48.71%.

Table 1:- Grading of biofilm formation in Pus isolates by the three different methods (N=78)

Biofilm Formation	TCP		TM		CRA	
	No.	%	No.	%	No.	%
High	19	24.36	14	17.95	1	1.28
Moderate	22	28.21	18	23.08	3	3.85
Weak/None	37	47.44	46	58.97	74	94.87

Table 1 shows the grading of bacterial biofilm formation in pus isolates by three different methods i.e. TCP, TM and CRA method into high, moderate and weak/none biofilm producers. Out of the three methods, TCP method detected strong biofilm production in maximum number of isolates 24.36%, whereas detection of strong biofilm production by TM and CRA methods was seen in 17.95% and 1.28% respectively (Figure 1). The TCP method had also detected more moderate biofilm producing bacteria 28.21% as compared to other methods i.e. 23.08% and 3.85% by the TM and CRA methods respectively.

Figure 1:- Grading of biofilm formation in Pus isolates by the three different methods

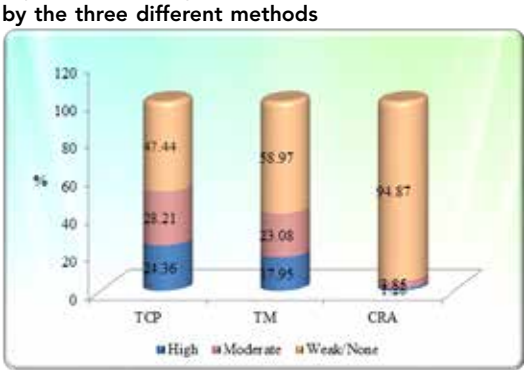

Table 2:- Comparison of detection of biofilm formation by three different methods

Total	TCP	TM	'p' Value*	CRA	'p' Value#
No. of biofilm producing isolates	41	32	0.199	4	<0.001
% of biofilm producing isolates	52.56	41.03		5.13	

*between TCP & TM
#between TCP & CRA
*Chi-square test

Table 2 shows comparison of detection of biofilm production by three different methods i.e. TCP, TM and CRA methods. Significant difference in biofilm detection was observed between CRA and TCP method (5.13% v/s 52.56%; p<0.05) whereas insignificant difference in biofilm detection was observed between TM and TCP (41.03% v/s 52.56%; p>0.05) (Figure 2).

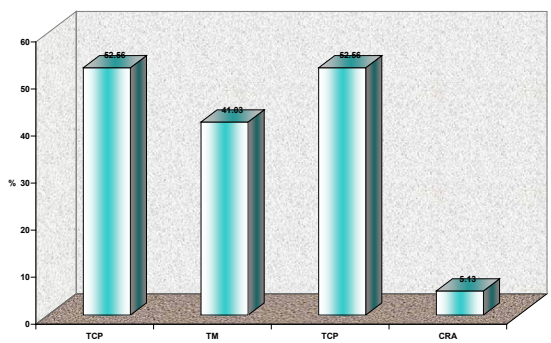

Figure 2:- Comparison of detection of biofilm formation by three different methods

Table 3:- Statistical evaluation of TM & CRA methods for detection of biofilm formation

Screening methods	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Diagnostic accuracy (%)
TM	78.05	100.00	100.00	80.43	88.46
CRA	9.76	100.00	100.00	50.00	52.56
'p' Value*	<0.001	NA	NA	0.002	<0.001

*'Z' test for difference of two proportions

Table 3 shows statistical evaluation of the different methods of biofilm detection. Considering the TCP method as gold standard for this study and comparing the data from TM and CRA methods, parameters like sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy were calculated. Tube method was 78.05% sensitive and 100.00% specific with PPV, NPV and accuracy of 100.00%, 80.43% and 88.46% respectively. CRA method was 9.76% sensitive and 100.00% specific with PPV, NPV and accuracy of 100.00%, 50.00 % and 52.56 % respectively (Figure 3).

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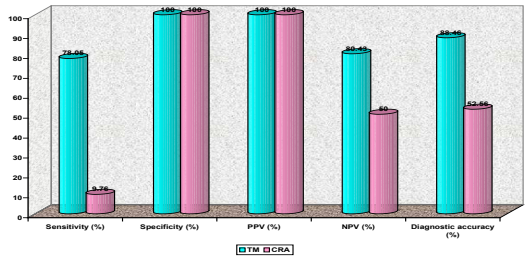

Figure 3:- Statistical evaluation of TM & CRA methods for detection of biofilm formation

Table 4:- Antibiotic resistance pattern of biofilm forming (B.F.) & Non-biofilm forming (N.B.F.) Staphylococci in Pus isolates (N=78)

Antibiotics	Resistance in B.F. isolates (N=41)		Resistance in N.B.F. isolates (N=37)		'p' Value*
	N	%	N	%	
Amoxiclav	20	48.78	17	45.95	0.981
Ciprofloxacin	25	60.98	14	37.84	0.069
Clindamycin	26	63.41	14	37.84	0.041
Erythromycin	24	58.54	12	32.43	0.025
Gentamycin	34	82.93	14	37.84	<0.001
Oxacillin	28	68.29	20	54.05	0.246
Doxycycline	30	73.17	23	62.16	0.425
Ticarcillin/Clavulanic acid	37	90.24	31	83.78	0.608
Vancomycin	0	0.00	0	0.00	NA
Linezolid	0	0.00	0	0.00	NA

Table 4 shows the comparison of the resistance pattern of biofilm forming (BF) and non-biofilm forming (NBF) Staphylococci in pus isolates. It shows that the biofilm producers are more resistant to the various antibiotics as compared to the non-biofilm producers. The BF bacteria showed 63.41% resistance to clindamycin, 58.54% to erythromycin, 82.93% to gentamycin as compared to 37.84%, 32.43% and 37.84% resistance respectively in NBF bacteria. This difference was significant (p value<0.05). With amoxiclav, ciprofloxacin, oxacillin, doxycycline and ticarcillin/clavulinic acid, 48.78%, 60.98%, 68.29%, 73.17% and 90.24% resistance respectively was observed among BF bacteria whereas NBF bacteria showed 45.95%, 37.84%, 54.05%, 62.16% and 83.78% resistance respectively. This difference was insignificant (p value>0.05), suggesting that resistance pattern was comparable between both the groups. The drugs that were 100% effective in both the groups were vancomycin and linezolid (Figure 4).

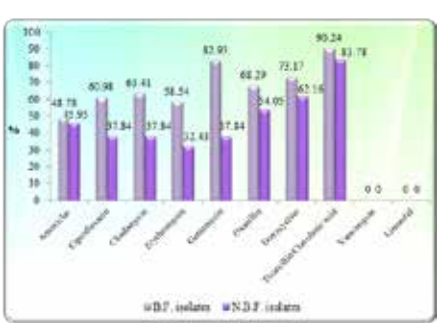

Figure 4:- Antibiotic resistance pattern of biofilm forming (B.F.) & Non-biofilm forming (N.B.F.) Staphylococci in Pus isolates

Discussion

Bacterial biofilm has profound implications for the host, as the sessile micro-organisms that are surviving in these matrix-enclosed aggregates are recalcitrant to antibiotic treatment and demonstrate persistence in spite of sustained host defenses. A number of microbial infections have been associated with surface colonization not only on live surfaces (Sinusitis, pulmonary infection in cystic fibrosis patients, periodontitis, etc. but also on medical implants (contact lenses, dental implants, intravascular catheters, urinary stents) etc. Biofilm formation represents a protected mode of growth that allows bacterial cells to stay alive in both hostile natural environments as well as in the human host, and enables them to disperse and colonize new niches whenever needed.[9]

In the present study, three different methods for detection of biofilm formation were used and their results were compared to find out the most appropriate method for demonstrating biofilm. Out of the 78 isolates, the TCP method detected biofilm in 41 isolates (52.56%), TM method detected biofilm in 32 isolates (41.03%) and CRA method detected biofilm in 4 isolates (5.13%). The present study showed the TCP method to be most sensitive for the biofilm detection, followed by the TM and CRA method.

Other authors have also reported TCP as the most sensitive method for biofilm detection. According to Mathur et al,[10] 53.94% were biofilm producers by TCP method, 41.44% by TM and 5.26% by CRA method.

Bose et al,[11] reported 54.18% biofilm producers by TCP method, 42.45% by TM method and 6.14% by CRA method.

Among the 110 isolates, tested by Hassan et al,[12] the TCP method showed biofilm in 63.63% isolates, tube method in 49.09% and CRA method in 10% isolates.

Tektook et al,[13] reported 60% biofilm producers by TCP method, 38% by TM method and 16% by CRA method.

Difference in the result of CRA method as shown in the above table may be due to variation in visual interpretation by different observers or due to difference in the composition of the media used for the study.

The current study was in concordance with most of the above studies. The TCP was found to be most sensitive method in detecting biofilm formation, followed by the TM and CRA method. The variation in the results of some of the studies in the above table may be due to the type of medium used, conditions of incubation, the nature of the solid surface and the difference in visual interpretation of the results by different observers.

In view of the large number of infections caused by biofilm producing bacteria, a reliable method for their diagnosis is necessary.

After completion of the study, we conclude that TCP method was the most reliable and sensitive method for biofilm detection and could assess both qualitatively and quantitatively, so we suggest that it can be used for accurate detection of biofilm producers by other laboratories.

In our study Vancomycin and Linezolid were found to be 100% sensitive against biofilm producing staphylococci. Since the drugs are very effective, relatively safe and can

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be used in patients of all ages, we suggest that these drugs can be used in treating staphylococcal biofilm infections.

Early detection of the biofilm producing organisms in the laboratory is necessary along with the determination of their antimicrobial susceptibility pattern, as these biofilm producing organisms are more resistant than their planktonic counterparts and usually do not respond to the conventional antibiotic therapy. Such infections are a major challenge for the physicians and have economic relevance as well. The antimicrobial susceptibility pattern will help the clinicians in prescribing appropriate antibiotics to the patients and thus prevent the emergence and spread of resistance.

References

1. Donlan RM, Costerton JW. Biofilms: the survival mechanisms of the clinically relevant microorganisms. Clin Microbiol Rev 2002;15:167-93.
2. Davies DG, Parsek MR, Pearson JP, Iglewski BH, Costerton JW, Greenberg EP. The involvement of cell to cell signals in the development of a bacterial biofilm. Science 1998;280:295-8.
3. Danhorn T, Fuqua C. Biofilm formation by plant associated bacteria. Annu Rev Microbiol 2007;61:401-22.
4. Toole GO, Kaplan HB, Kolter R. Biofilm formation as microbial development. Annu Rev Microbiol 2000;54:49-79.
5. Donlan RM. Biofilm and device associated infections. Emerg Infect Dis 2001; 7(2),277-281.
6. Christensen GD, Simpson WA, Younger JJ, et al. Adherence of coagulase negative Staphylococci to plastic tissue culture plates: a quantitative model for the adherence of Staphylococci to medical devices. J Clin Microbiol 1985;22(6):996-1006.
7. Christensen GD, Simpson WA, Bisno AL, Beachey EH. Adherence of slime producing strains of *Staphylococcus epidermidis* to smooth surfaces. Infect Immun 1982;37(1):318-326.
8. Freeman DJ, Falkiner FR, Keane CT. New method for detecting slime production by coagulase negative Staphylococci. J Clin Pathol 1989;42 (8):872-4
9. Verma P, Singh S. Clinical distribution of Biofilm Forming *Staphylococcus aureus* and its sensitivity against some antibiotics. World Journal of Pharmacy and Pharmaceutical Sciences 2015;4(4):942-952
10. Mathur T, Singhal S, Khan S, Upadhyay D J, Fatma T, Rattan A. Detection of biofilm formation among the clinical isolates of Staphylococci: An evaluation of three different screening methods. Indian J Med Microbiol 2006;24:25-9.
11. Bose S, Khodke M, Basak S, Mallick SK. Detection of Biofilm producing Staphylococci: Need of the hour. J. Cin. Diagnostic Res 2009;3(6):1915-1920.
12. Hassan A, Usman J, Kaleem F, Omair M, Khalid A, Iqbal M. Evaluation of different detection methods of biofilm formation in the clinical isolates. Braz J Infect Dis. 2011;15(4):305-11.
13. Tektook NK, Essa RH, Hussein SS. Evaluation of genotype and phenotype methods for detection of Biofilm formed by *Staphylococcus epidermidis* isolated from patients in some hospitals of Baghdad city. World Journal of Pharmaceutical Research 2015;4(3):1835-1854.