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Comparison Of Antimicrobial Susceptibility Pattern Of Biofilm Producing And Non-Biofilm ProducingStaphylococci Isolated From Various Clinical Samples Of Patients From A Tertiary Care Hospital

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ABSTRACT

Biofilms have great significance in medical field as they decrease susceptibility to antimicrobial agents. We tested 242 clinical isolates of staphylococci for biofilm production by Tissue culture plate method (TCP) and compared the Antimicrobial Susceptibility Pattern of biofilm producing and non-biofilm producing staphylococci. Association of Multidrug Resistance with biofilm formation was also observed. Further speciation of biofilm positive staphylococci was done by MicroScan system. Biofilm producers were 142 (58.6%) out of 242 staphylococcal isolates, of which 81 (57.04%) biofilm producers were CNS and 61 (42.96%) were CPS. Maximum biofilm production was seen by S.epidermidis isolates i.e. 72 (50.70%), followed by 61 isolates (42.96%) of S.aureus, 5 (3.52%) isolates of S.haemolyticus, and 2 isolates (1.41%) of S.lugdunensis. Only 1 isolate (0.70%), each of S.saprophyticus and S.schleiferi formed biofilm. The biofilm producing staphylococcal isolates showed higher resistance to ciprofloxacin, clindamycin, erythromycin and gentamicin as compared to non-biofilm producing staphylococcal isolates. Vancomycin and linezolid were 100% effective drugs against both groups. A higher prevalence of Multidrug resistant isolates was observed among the biofilm producers (92.95%) than the non-biofilm producers (60%). In our study Vancomycin and linezolid were found to be 100% sensitive against biofilm producing staphylococci, hence we suggest that these drugs can be used in treating staphylococcal biofilm infections.

KEYWORDS biofilm, staphylococci, Multidrug Resistance

Introduction-

Biofilms are microbial communities characterized by cells that are irreversibly attached to a substratum or interface or to each other, embedded in a self-produced extracellular polymeric matrix.[1] Biofilms have great significance in medical field as they decrease the susceptibility to the antimicrobial agents. They also facilitate plasmid exchange, thereby enhancing the spread of antimicrobial resistance. The proximity of cells within the biofilm facilitate this plasmid exchange.[2] It has been seen that 65% of the nosocomial infections in human beings are associated with biofilms, which may increase the cost of the medical health care upto billions of dollars as these biofilm infections are 10 to 1000 times more resistant to the effects of the antimicrobial agents. It is believed that the mechanism responsible for this enhanced antimicrobial resistance involves alterations in gene expression which lead to a phenotype difference between the planktonic and sessile forms. The sessile forms are more resistant as they produce exopolysaccharide, have different growth characteristics and take up the nutrients and drugs differently from their planktonic counterpart.[3]

Material and Methods-

The present study was conducted on 242 non-repetitive clinical isolates of staphylococci obtained from various clinical samples of patients admitted in the various wards and intensive care units of the hospital, over a period of one year from June 2014 to May 2015, in the Department of Microbiology, National Institute of Medical Sciences & Research, Nims University, Jaipur (Rajasthan). Clinical samples like pus, urine, sputum and indwelling medical devices like catheter tips (urinary catheter tips, central venous catheter tips and intravenous catheter tips) were collected. Samples were collected with all aseptic precautions and transported to the microbiology laboratory and cultured as per standard protocol. All isolates of Staphylococcus were then further tested for detection of biofilm production by Tissue culture plate method (TCP) and classified as biofilm producing and non-biofilm producing staphylococci . Antimicrobial susceptibility testing was done on Mueller Hinton agar by Kirby Bauer disk diffusion method.[4] Further speciation of biofilm positive staphylococci was done by MicroScan system (Siemens MicroScan autoSCAN4).

Tissue Culture Plate Method (TCP)

The TCP assay described by Christensen et al.[5] 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 (visible turbidity) 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 were 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.

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

Results-

Out of 242 Staphylococcal isolates, maximum isolates were obtained from catheter tip i.e. 106 (43.80%) followed by 78 isolates (32.23%) from pus and 42 isolates (17.36%) from urine. The minimum number of isolates were obtained from sputum i.e. 16 (6.61%). Out of 242 Staphylococci, 128 (52.89%) were coagulase negative Staphylococci (CNS) and 114 (47.11%) coagulase positive Staphylococci (CPS). TCP method detected 142 (58.6%) staphylococcal isolates as biofilm producers out of 242

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staphylococcal isolates. Out of the 142 isolates, 81 (57.04%) biofilm producers were CNS and 61 (42.96%) were CPS. Biofilm production was observed maximally 69 (65.09%) in catheter tip isolates, followed by 24 (57.14%) in urine isolates, 41 (52.56%) in pus isolates and 8 (50.00%) in sputum isolates.

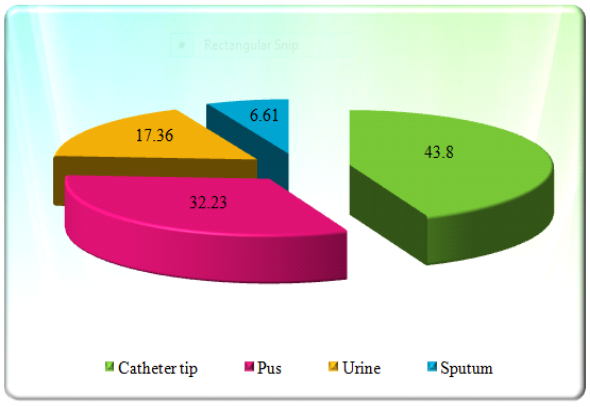

Figure 1:- Distribution of Staphylococcal isolates in different samples

Table 1:- Detection of Biofilm Producers in the total isolates by Tissue Culture Plate method

Source	No.	%
Catheter tip (N=106)	69	65.09
Pus (N=78)	41	52.56
Urine (N=42)	24	57.14
Sputum (N=8)	8	50.00

Table 1 shows biofilm production of the isolates by TCP method. Biofilm production was observed maximally 69 (65.09%) in catheter tip isolates, followed by 24 (57.14%) in urine isolates, 41 (52.56%) in pus isolates and 8 (50.00%) in sputum isolates.

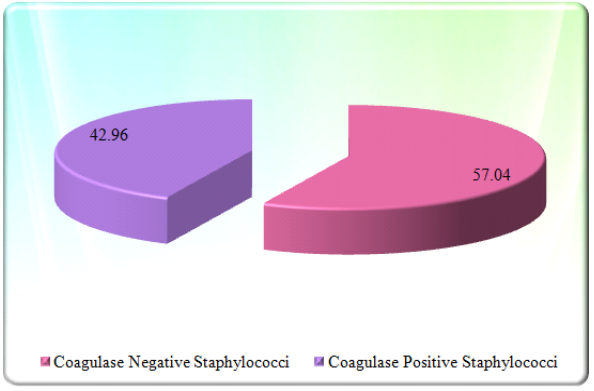

Figure 2:- Biofilm producing Staphylococci (N=142)

Table 2:- Grading of biofilm formation in total isolates by the Tissue Culture Plate (N=242)

Biofilm Formation	TCP	
	No.	%
High	68	28.10
Moderate	74	30.58
Weak/None	100	41.32

Table 3:- Biofilm production of Staphylococci with regards to the source of isolates & Coagulase reaction

Source	CPS				CNS			
	B.F.		N.B.F.		B.F.		N.B.F.	
	No.	%	No.	%	No.	%	No.	%
Catheter tip (N=106)	21	19.81	16	15.09	48	45.28	21	19.81
Pus (N=78)	26	33.33	23	29.49	15	19.23	14	17.95
Urine (N=42)	9	21.43	8	19.05	15	35.71	10	23.81
Sputum (N=8)	5	31.25	6	37.50	3	18.75	2	12.50

Table 4:- Antibiotic resistance pattern of biofilm forming (B.F.) & Non-biofilm forming (N.B.F.) Staphylococci in total isolates (N=242)

Antibiotics	Resistance in B.F. isolates (N=142)		Resistance in N.B.F. isolates (N=100)		'p' Value*
	N	%	N	%	
Amoxiclav	85	59.86	53	53.00	0.353
Ciprofloxacin	101	71.13	38	38.00	<0.001
Clindamycin	107	75.35	38	38.00	<0.001
Erythromycin	96	67.61	43	43.00	<0.001
Gentamycin	121	85.21	41	41.00	<0.001
Oxacillin	100	70.42	58	58.00	0.063
Doxycycline	100	70.42	60	60.00	0.121
Ticarcillin/Clavulanic acid	117	82.39	74	74.00	0.157
Vancomycin	0	0.00	0	0.00	NA
Linezolid	0	0.00	0	0.00	NA

Chi-square test

Table 4 shows the comparison of the resistance pattern of biofilm forming and non biofilm forming Staphylococci in total 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 71.13% resistance to ciprofloxacin, 75.35% to clindamycin, 67.61% to erythromycin and 85.21% to gentamycin as compared to 38.00%, 38.00%, 43.00% and 41.00% resistance respectively in NBF bacteria. This difference was significant (p value<0.05). With amoxiclav, oxacillin, doxycycline and ticarcillin/clavulinic acid, 59.86%, 70.42%, 70.42%, and 82.39% resistance respectively was observed among BF bacteria whereas NBF bacteria showed 53.00%, 58.00%, 60.00% and 74.00% 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.

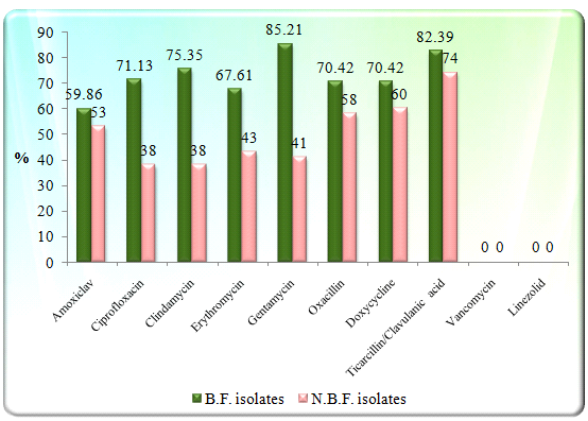

Figure 3:- Antibiotic resistance pattern of biofilm forming (B.F) & Non-biofilm forming (N.B.F) Staphylococci in total isolates

Table 5:- Comparison of Multidrug Resistance (MDR) among the Biofilm Forming (B.F.) & Non Biofilm Forming (N.B.F.) strains

Sources	No. of B.F. isolates	B.F. MDR isolates		No. of N.B.F. isolates	N.B.F. MDR isolates		'p' Value*
	N	N	%	N	N	%	
Catheter tip	69	67	97.10	37	21	56.75	<0.001
Pus	41	34	82.92	37	21	56.75	0.022
Urine	24	23	95.83	18	12	66.66	0.036
Sputum	8	8	100	8	6	75.00	0.450
Total	142	132	92.95	100	60	60	<0.00

*Chi-square test

Table 5 shows comparison of MDR among the BF and NBF isolates. MDR isolates were found more among the BF bacteria (92.95%) as compared to NBF bacteria (60%). There was significant difference in MDR between BF and NBF isolates in catheter tip, pus and urine isolates ($p<0.05$). There was no significant difference in MDR between BF and NBF isolates in sputum isolates (100% v/s 75.00%; $p>0.05$).

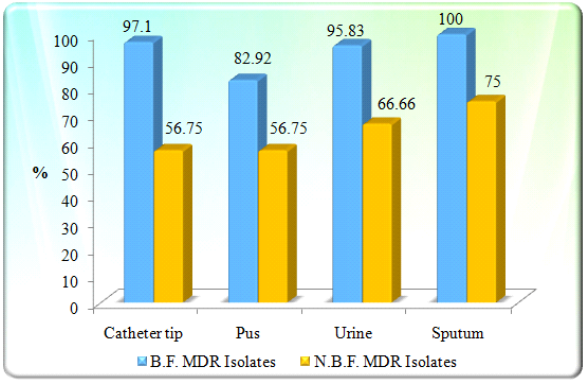

Figure 4:- Multidrug Resistance (MDR) among the Biofilm Forming (B.F) & Non-Biofilm Forming (N.B.F) strains

Table 6:- Speciation of biofilm forming Staphylococci by MicroScan System

Organism	Biofilm Producer by TCP	Percentage %
S. epidermidis	72	50.70
S. aureus	61	42.96
S. haemolyticus	5	3.52
S. lugdunensis	2	1.41
S. saprophyticus	1	0.70
S. schleiferi	1	0.70
Total	142	100.00

Table 6 depicts speciation of biofilm forming Staphylococci by MicroScan. Maximum biofilm production was seen by *S.epidermidis* isolates i.e. 72 (50.70%), followed by 61 isolates (42.96%) of *S.aureus*, 5 (3.52%) isolates of *S.haemolyticus,* and 2 isolates (1.41%) of *S.lugdunensis*. Only 1 isolate (0.70%), each of *S.saprophyticus* and *S.schleiferi* formed biofilm.

Discussion-

In the present study, TCP method detected 142 (58.6%) staphylococcal isolates as biofilm producers out of 242 staphylococcal isolates. Nearly similar results were obtained by different authors. Mathur et al[6] reported 82 (53.9%) biofilm producers out of 152 total isolates, Knobloch et al[7] reported 73 (57.1%) biofilm producers out of 128 total isolates , Bose et al[8] reported 97 (54.1%) biofilm producers out of 179 total isolates and Verma et al[9] reported 22 (57.8%) biofilm producers out of 38 total isolates. However, Oliviera et al[10] and Garima et al[11] reported 81 (81%) biofilm producers out of 100 total isolates and 59 (84.2%) biofilm producers out of 70 total isolates, respectively which is higher than the present study. This variation in the biofilm production may be due to difference in the prevalence of biofilm producer in different hospital and different geographical area, in addition to difference in methods of isolation.

We compared the antibiotic sensitivity pattern of the biofilm forming (B.F) and the non-biofilm forming (NBF) staphylococcal isolates, in the present study. The biofilm producing staphylococcal isolates showed higher resistance to ciprofloxacin, clindamycin, erythromycin and gentamicin as compared to non-biofilm producing staphylococcal isolates. Resistance to amoxiclav, oxacillin, doxycycline and Ticarcillin/clavulinic acid was similar in both the groups ($p>0.05$).

In the present study, all the staphylococcal isolates (BF and NBF) were sensitive to Vancomycin and linezolid. Similar findings were observed in the studies of Hassan et al,[12] Garima et al[11] and Khan et al,[13] who reported that all the staphylococcal isolates were sensitive to Vancomycin and linezolid.

We also found significant difference in the prevalence rate of MDR isolates among the biofilm producing and non-biofilm producing staphylococcal isolates. MDR isolates were found more among the BF bacteria (92.95%) as compared to NBF bacteria (60%). Sarita Yadav et al[14] and Sanchez et al[15] in their study also, reported a higher prevalence of MDR isolates among the biofilm producers than the non-biofilm producers. A higher antibiotic resistance in biofilm producing bacteria than non-biofilm bacteria was also observed in the study of Rewatkar et al.[16]

The increased resistance of biofilm producing strains to antibiotics may be due to impaired penetration of the drug across the biofilm, expression of resistance genes and also because the biofilm bacteria exhibit a slow rate of metabolism and divide infrequently resulting in decreased sensitivity to antibiotics targeted at cell wall synthesis.

Out of 142 biofilm forming isolates of staphylococci, the most common species isolated was *S.epidermidis* i.e. 50.70% followed by *S.aureus* which was 42.96%. This is in concordance with the study of Bose et al,[8] who obtained 57.55% *S.epidermidis* isolates and 42.44% *S.aureus* isolates out of 139 biofilm producing staphylococcal isolates. Terki et al,[17] in their study obtained 33.33% biofilm forming strains of *S.epidermidis* and 27.77% biofilm forming strains of *S.aureus*, which is lower than the present study. Biofilm forming strains of *S.haemolyticus* and *S.lugdunensis* were 3.52% and 1.41% respectively, in the present study whereas only 0.70% strains of *S.saprophyticus* and *S.schleiferi*, formed biofilm. Sharvari et al,[18] in their study reported that, out of 172 biofilm producers, 4.65% strains were *S.haemolyticus*, 1.74% were *S. lugdunensis* and *S.schleiferi*, which is comparable with the present study.

Conclusion-

Biofilms have been recognised as being important in human disease and the number of biofilm-associated diseases seems to be increasing. As medical interventions rely increasingly on medical devices and prosthesis, the need to prevent, reduce, or eliminate microbial biofilms is becoming an important constraint. The tendency of microorganisms to develop biofilms has been well documented for a number of medical devices. This process is particularly relevant for the clinician because biofilm associated microorganisms are much more resistant to antimicrobial agents than the planktonic organisms. If antimicrobial therapy is considered a viable option against biofilm colonization, then susceptibility testing should be performed with biofilm-associated organisms. The antimicrobial susceptibility pattern will help the clinicians in prescribing appropriate antibiotics to the patients and

thus prevent the emergence and spread of resistance. In our study, TCP method was found to be a reliable and sensitive method for biofilm detection, that can assess both qualitatively and quantitatively, hence it can be used for accurate detection of biofilm producers by other laboratories. Since Vancomycin and linezolid were found to be 100% sensitive against biofilm producing staphylococci in our study, we suggest that these drugs can be used in treating staphylococcal biofilm infections.

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