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Original Research Article

Biofilm formation by bacteria isolated from intensive care units of a tertiary care hospital, with special relevance to its risk factors

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ABSTRACT

Background: The purpose of this study was to detect biofilm formation by bacterial isolates from patients with device associated infection admitted in intensive care units (ICUs), to compare the three methods used for detection of bioiflm, to compare the antimicrobial susceptibility pattern of the biofilm producers with the non-producers and to study the risk factors associated with biofilm formation.
Methods: A total of 115 bacterial isolates from patients with device associated infection admitted in different ICU for a period of one year was included in the study. These clinical isolates were detected for biofilm formation by tissue culture plate method, tube method and Congo red agar method. Kirby-Bauer disc diffusion method of antibiotic susceptibility was performed on all isolates.
Results: Out of the 115 bacterial isolates, 71 were biofilm producers. Tissue culture plate method detected the maximum number of biofilm producers (61.7%). The maximum number of biofilm producers were isolated from tracheal aspirate and endotracheal tubes (52.1%) followed by blood (17%) and urine (12.6%) respectively. The predominant biofilm producing isolates were *Klebsiella pneumoniae* (39.4%), *Staphylococcus aureus* (19.7%) and *Pseudomonas aeruginosa* (16.9%). Multi drug resistance among the biofilm producers was significantly higher than the non-biofilm producers (p value=0.0125). The risk of biofilm formation was seen to increase with the increase in duration of hospital stay (p value=0.0092, statistically very significant).
Conclusions: From this study it was found that a high degree of biofilm producers were isolated from patients on indwelling devices. Tissue culture plate was found to be the most accurate method. The degree of multidrug resistance among the bioiflm producers was significantly higher than the non-producers.

Keywords: Antibiotic susceptibility pattern, Biofilms, Congo red agar method, Intensive care units, Tissue culture plate method, Tube method

INTRODUCTION

Bacterial biofilms create significant obstacles in both medical and industrial settings. In the medical field, it is estimated that biofilms cause over 80% of microbial infections in the body and approximately 65% of nosocomial infections involve biofilms. Biofilms are increasingly being recognized by the public health community as an important source of bacterial pathogens especially in those with indwelling medical devices. They contribute significantly to the emergence and dissemination of antibiotic resistance traits in the nosocomial setting thus causing persistent or recurrent infections.[1] Biofilms are complex communities of single or multiple species of microorganisms that develop on abiotic (e.g. rocks) and biotic (host mucosal tissue) surfaces. Bacterial cells in biofilm micro colonies are held together by slime matrix i.e. extracellular polysaccharides (EPS).[2] Biofilm associated microorganisms behave differently from planktonic (freely suspended) organisms with respect to growth rates and ability to resist antimicrobial treatments and therefore

pose a public health problem.[3] Biofilms are associated with many medical conditions including indwelling medical devices, dental plaque, upper respiratory tract infections, peritonitis, and urogenital infections.[4] It has been found that 95% of the urinary tract infections were associated with urinary catheters, 86% pneumonias were associated with mechanical ventilation and 85% of the blood stream infections were closely related to intravascular devices.[5]

The objective of this study was to detect biofilm formation by bacterial isolates from patients with device associated infection admitted in intensive care units (ICUs), to compare the three methods used for detection of biofilm, to compare the antimicrobial susceptibility pattern of the biofilm producers with the non-producers and to study the risk factors associated with biofilm formation.

METHODS

It was a prospective study conducted at the Department of Microbiology, Gauhati Medical College and Hospital for a period of one year from June 2011 to May 2012.

Selection criteria

Samples from patients who were on indwelling devices for more than 48 hours with clinical diagnosis of device associated urinary tract infections, blood-stream infection and pneumonia from different intensive care units (ICUs) were taken.

Procedure

A total of 115 clinical isolates were recovered from the 105 clinical specimens.

Under all aseptic conditions following clinical specimens were collected i.e., blood, urine from urinary catheter, endotracheal tubes, tracheal aspirates, central line tips, drainage catheter.

In case of central line tips, roll-plate technique was used. Here the catheter tip was rolled over the surface of blood and MacConkey agar with the help of a sterile forceps and incubated aerobically at 37°C. The plates were examined after 24 hours and if no growth, reincubated and examined again after 48 hours.[6] Catheter-tip infection has been defined as microbial growth of ≥15 cfu per semiquantitative agar plate.[6]

Urine samples were inoculated on blood agar, MacConkey's agar and Cystine lactose electrolyte deficient (CLED) medium with a calibrated loop to determine colony forming units (CFU). All specimens with bacteriuria of >103 colony forming units (cfu)/ml urine (which defines CAUTI) of one or two organisms were analysed.[7] Isolates were identified by standard microbiological procedures. All the isolates were subjected to antimicrobial susceptibility testing by Kirby-Bauer disc diffusion method.

Tests for biofilm production

Control strains: Biofilm-producing reference strain of *Pseudomonas aeruginosa* (ATCC 27853) and non-biofilm forming reference strains of *Staphylococcus aureus* (ATCC 25923) and *Escherichia coli* (ATCC 25922) were used.[8]

Tissue culture plate method:[9]

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. Turbidity of the suspension was adjusted to 0.5 McFarland standard. The culture was further diluted 1:100 with fresh medium. 96 wells flat bottom tissue culture plates (Himedia Pvt Ltd, Mumbai, India) 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 of 450 nm. The average OD values were calculated for all tested strains and negative controls, the cut-off value (ODc) was established. It is defined as a three standard deviations (SD) above the mean OD of the negative control: ODc=average OD of negative control + (3×SD of negative control). The tests were performed in duplicate. For easier interpretation of the results, strains were divided into the following categories: 1. Non biofilm producer (0) OD ≤ODc; 2. Weak biofilm producer (+ or 1) = ODc <OD ≤2×ODc; 3. Moderate biofilm producer (++ or 2) = 2×ODc <OD≤4×ODc; 4. Strong biofilm producer (+++or 3), 4×ODc <OD.

Tube method:[10]

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. Results were read as absent, weak, moderate and strong.

Congo red agar method:[11]

The medium was composed of brain heart infusion broth (Oxoid Ltd, Basingstoke, England) 37 gm/l, sucrose 50 gm/l, agar No. 1 (Oxoid) 10 gm/l and Congo red (BDH Ltd) 08 gm/l. Congo red stain was prepared as a concentrated aqueous solution and autoclaved (121°C for 15 minutes) separately from the other medium constituents, and was then added when the agar had cooled to 55°C. Plates of the medium were inoculated and incubated aerobically for 24 hours at 37°C. A positive result was indicated by black colonies with a dry crystalline consistency. Non-slime producers usually remained pink, though occasional darkening at the centre of the colonies was observed and this gave a bull's eye appearance. An indeterminate result was indicated by a darkening of the colonies but with the absence of a dry crystalline colonial morphology.

Ethical approval

Approval from the Institutional Ethics Committee was obtained before conducting the study.

Statistical analysis

All collected data were statistically analyzed using statistical software INSTAT. The results are presented as number and percentage and chi-square test was done wherever necessary to calculate the p value. The p value of less than 0.05 was considered to be statistically significant.

RESULTS

A total of 115 clinical isolates obtained from 105 clinical specimens received from the different ICU's of GMCH over a period of 1 year were studied. The study included clinical samples from five different types of ICUs i.e. emergency ICU (EICU), general ICU (GICU), intensive therapy unit (ITU), neonatal ICU (NICU), and paediatric ICU (PICU). Tracheal aspirates and endotracheal tubes together contributed a majority of the clinical specimen 45.7% followed by blood 24.8%.

Table 1: The total number of biofilm producers detected by TCP, TM and CRA.

Biofilm detection methods	Total number of biofilm producers (positive) (%)	Total number of non-producers (negative) (%)
Tissue culture plate (TCP)	71 (61.7)	44 (38.2)
Tube method (TM)	48 (41.7)	67 (58.2)
Congo red agar (CRA)	21(18.2)	94 (81.7)

Table 1 shows that TCP detected the highest number of biofilm producers i.e. 61.7% whereas the CRA method detected the lowest number of biofilm producers 18.2%. The TM detected 41.7% biofilm producers. Table 2 shows that TCP further categorized the biofilm producers as strong 4.2%, moderate 11.2% and weak 84.5% producers. In the tube method 6.2% were strong producers, the number of moderate and weak producers being 35.4% and 58.3% respectively.

Table 2: Comparison of TCP and TM method for detection of biofilm producers.

Number of isolates	Biofilm formation	Tissue culture plate (N=71) (%)	Tube method (N=48) (%)
Clinical isolates (N=115)	Strong	3 (4.2)	3 (6.2)
	Moderate	8 (11.2)	17 (35.4)
	Weak	60 (84.5)	28 (58.3)
	Non	44 (38.2)	67 (58.2)

Table 3: The quantitative analysis of biofilm production by clinical isolates as evaluated by tissue culture plate method.

Organisms Isolated	Total no. of isolates N=115 (%)	Total no. of biofilm producers N=71 (%)	Strong producers N=3 (%)	Moderate producers N=8 (%)	Weak producers N= 60 (%)	Non biofilm producers N=44 (%)
Staphylococcus aureus	24 (20.8)	14 (19.7)		2 (25)	12 (20)	10 (22.7)
Staphylococcus epidermidis	8 (6.9)	5 (7)			5 (8.3)	3 (6.8)
Enterococcus sp.	4 (3.4)	3 (4.2)			3 (5)	1 (2.2)
Klebsiella pneumoniae	39 (33.9)	28 (39.4)	2 (66.6)	2 (25)	24 (40)	11 (25)
Escherichia coli	11 (9.5)	7 (9.8)	1 (33.3)	-	6 (10)	4 (9)
Citrobacter sp	1 (0.08)	0				1 (2.2)
Pseudomonas aeruginosa	23 (20)	12 (16.9)	-	4 (50)	8 (13.3)	11 (25)
Proteus mirabilis	3 (2.6)	1 (1.4)	-	-	1 (1.6)	2 (4.5)
Acinetobacter baumanni	2 (1.7)	1 (1.4)	-	-	1 (1.6)	1 (2.2)
Total	115	71	3	8	60	44

The maximum number of biofilm producers was isolated from tracheal aspirates and endotracheal tubes 52.1% followed by blood 17% and urine 12.6% respectively. *Klebsiella pneumoniae* 39.4% was the highest biofilm producing isolates followed by *Staphylococcus aureus* 19.7%. *Proteus mirabilis* 1.4% and *Acinetobacter baumannii* 1.4% were the least biofilm producers (Table 4).

Table 4: Comparison of the antibiotic susceptibility pattern of biofilm producing and non-biofilm producing enterobactericiae.

Antibiotics tested	Biofilm producing isolates (N=36) (%)		Non biofilm producing isolates (N=18) (%)	
	Sensitive	Resistant	Sensitive	Resistant
Ampicillin	-	100	-	100
Ciprofloxacin	5.5	94.5	29.4	70.6
Levofloxacin	5.5	94.5	29.4	70.6
Amikacin	11.1	88.9	29.4	70.6
Imipenem	61.1	38.9	94.1	5.9
Pipercillin-tazobactum	41.7	58.3	47.1	52.9
Ceftriaxone	16.6	83.4	29.4	70.6
Cefepime	16.6	83.4	29.4	70.6

Comparison of antimicrobial susceptibility pattern of biofilm producers and biofilm non-producers

Enterobacteriaceae, *Pseudomonas aeruginosa* and other non-fermenting organisms (*Acinetobacter sp.*) resistant to three or more of the following antibiotic classes: cephalosporins, penicillins, fluoroquinolones and aminoglycosides were considered to be multidrug resistant (MDR).

On studying the association between the biofilm producers and multidrug resistance, a significant association was found (p value: 0.0125 by Fisher exact test).

Out of the 13 biofilm producing non-lactose fermenters isolated in the study, 92.3% (12/13) were MDR whereas only 50% (6/12) non producers were MDR (p value=0.0302, considered significant). High degree of resistance was seen towards ceftriaxone (94.2%), cefepime (94.2%), amikacin (88.3%), ciprofloxacin (82.4%) and pipercillin-tazobactum (53%). Imipenem resistance was found to be 53.8% (7/13) among the biofilm producers as compared to 25% (3/12) among the non-producers.

Out of the 36 biofilm producing enterobactericiae isolates, 72% (26/36) were MDR whereas only 57.1% (8/14) non-biofilm producers were multidrug resistant (p value=0.33, not significant). Imipenem resistance was found to be 36.1% (13/36) among the biofilm producers as compared to 7.1% (1/14) among the non-producers. High degree of resistance was seen against ciprofloxacin (94.5%), amikacin (88.9%), ceftriaxone (83.4%), cefepime (83.4%), pipercillin-tazobactum (58.3%) among the biofilm producers (Table 5).

Table 5: Comparing the antibiotic sensitivity pattern of biofilm producing and non-biofilm producing gram positive bacteria.

Antibiotics used	Biofilm producing isolates (%)		Non biofilm producing isolates (%)	
	Sensitive	Resistant	Sensitive	Resistant
Penicillin G	4.3	95.7	7.6	92.4
Methicillin	13	87	23	77
Cotrimoxazole	4.3	95.7	15.3	84.7
Erythromycin	4.3	95.7	7.6	92.4
Vancomycin	100	-	100	-
Levofloxacin	21.7	78.3	30	70
Gentamycin	43.4	56.6	61.5	38.5
Imipenem*	NT	NT	NT	NT

NT: Not Tested

Out of the 24 *Staphylococcus aureus* isolates obtained in the study, 50% were MRSA as well as biofilm producers, whereas among the non-biofilm producers, only 33% were MRSA. No VRE were detected in this study.

Statistical analysis of tissue culture plate, tube and Congo red agar methods

Considering 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 TCP, TM and CRA. False positive were biofilm producers by TM and CRA method but not by TCP method. False negatives were non-biofilm producers by TM and CRA methods but the same strains were biofilm producers by TCP method. True negatives were non-biofilm producers by all the methods. The sensitivity, specificity, positive predictive value, negative predictive value of TM were 57.8%, 89.7%, 91.6% and 52.2% respectively. The sensitivity, specificity, positive predictive value, negative predictive value of CRA method were 21.9%, 85%, 72.7% and 37.3% respectively.

Table 6 shows the risk factors for formation of biofilm in patients with indwelling devices. It is seen that the risk of biofilm formation increases with the increase in duration of hospital stay, p value=0.0092, which is considered very significant.

Table 6: The risk factors associated with biofilm formation in indwelling devices.

Risk Factors	Total isolates n=115 (%)	Biofilm producing isolates (%) n=71	Statistical analysis (p value)
Duration of hospital stay			
<10 days	42 (36.5)	19 (45.2)	P value=0.0092 (very significant)
10-20 days	69 (60)	49 (71)	
>20 days	4 (3.4)	3 (75)	
Duration of stay in ICU			
<10 days	64 (55.6)	38 (59.3)	P value=0.569(not significant)
10-20 days	48(41.7)	31 (64.5)	
>20 days	3(2.6)	2 (66.6)	
Duration of indwelling device (ET, CVC, urinary catheter tip, chest tube tip)	n= 69	n=50	
<10 days	54 (78.2)	38 (70.3)	P value=0.533 (not significant)
10-20 days	15 (21.7)	12 (80)	
>20 days	-	-	
Site of insertion of CVC			
Jugular vein	9 (7.8)	6 (66.6)	P value=1 (not significant)
Subclavian vein	5 (4.3)	3 (60)	

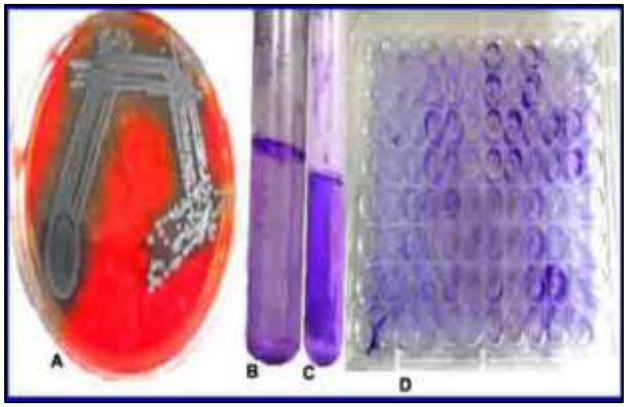

Figure 1: A) Showing a positive biofilm producing isolate with black colonies on Congo red agar plate; B) showing moderate biofilm producer by tube method; C) showing strong bioiflm producer by tube method; D) showing biofilm producers in tissue culture plate method.

DISCUSSION

Bacterial biofilm has long been considered as a virulence factor contributing to infection associated with various medical devices and causing nosocomial infection. As the use of indwelling medical devices (IMDs) increases, the incidence of biofilm-related infections represents a current and growing unmet medical need. Catheter colonization by biofilm producing microbes is a crucial step in ensuing catheter-related sepsis.[12] If an indwelling medical device is colonized by a biofilm, the problem will inevitably get worse, and the aging biofilm will become increasingly difficult to treat against. Old biofilms have been shown to be even less susceptible to antimicrobial agents than are younger biofilms. In addition, if organisms with acquired resistance are present in the biofilm, the probability of resistance plasmid transfer might increase over time.[13] The TCP method detected 61.7% biofilm producers and 38.2% were non biofilm producers. TCP further categorized the biofilm producers as strong 4.2%, moderate 11.2% and weak 84.5% producers. The findings are similar to a study done by Hassan et al where the number of isolates showing biofilm formation in the TCP method was 64.7%, and non-biofilm producers were 36.3%.[14] Bose et al found that biofilm formation in TCP method was 54.19% and non-biofilm producers were 45.81%.[15] In the tube method 41.7% were biofilm producers and 58.2% were non-producers. 6.2% were strong producers; the number of moderate and weak producers being 35.4% and 58.3% respectively. The findings of the study are similar to that of Hassan et al in which the tube method detected 49% isolates as biofilm producers and 51% as non-biofilm producers.[14] The CRA method could detect only 18.2% biofilm producers and 81.7% were non-producers. The findings of CRA method were similar to a study done by Eftekhar et al where the number of biofilm producers in CRA method was 24%.[16] Bose et al reported that in CRA, 6.15% strains produced biofilm and 93.85% were non-biofilm producers.[15] The use of CRA yields inconsistent results and shows very little correlation with either TCP or TM. In our study as well, the diversity in colony colours was variable and sometimes it was difficult to differentiate. Therefore this study does not recommend CRA for screening biofilm producers.

Out of the 13 biofilm producing non-lactose fermenters isolated in the study, 92.3% were MDR whereas only 50% non-producers were MDR. Imipenem resistance was found to be 53.8% among the biofilm producers as compared to 25% among the non-producers. Kalaivani et al also found a similar finding of 93% MDR

Pseudomonas sp among the biofilm producers.[17] In a study done by Dheepa et al similar finding of 54% Imipenem resistance was seen among the biofilm producers.[18]

Out of the 24 *Staphylococcus aureus* isolates obtained in the study, 50% were MRSA as well as biofilm producers, whereas among the non-biofilm producers, only 33% were MRSA. In a similar study done by Agarwal et al, 53.7% MRSA isolates were biofilm producers while only 17.6% of MRSA isolates were non-biofilm producers.[19]

A highly significant correlation existed between the ability of the strains to form biofilms and antimicrobial resistance. There may be various factors responsible for increased antimicrobial resistance among the biofilm producers. It may be due to the fact that ours is a tertiary care hospital with widespread usage of broad spectrum antibiotics leading to selective survival advantage of pathogen. Other possible reasons might be delayed penetration of antimicrobial agent through the biofilm matrix, altered growth rate of organisms in biofilms and other physiological changes due to biofilm mode of growth.

The sensitivity, specificity, positive predictive value, negative predictive value of CRA method were 21.9%, 85%, 72.7% and 37.3%. Similarly, in a study done by Hassan et al sensitivity and specificity of TM was 73% and 92.5%, respectively.[14]

Molecular detection of genes related to biofilm formation could not be done which is a limitation of this study.

CONCLUSION

From this study it can be concluded that a high degree of biofilm producers were isolated from ICU patients on various indwelling devices. Antibiotic resistance and multidrug resistance among the bioiflm producers were significantly higher than the non-producers. Out of the three methods used for detection of biofilms, TCP method could detect the maximum number of biofilm producers.

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