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Original Research Article

Biofilm Formation among Bacteria Isolated from Different Types of Human Infections

Riti Jain Seth[1]*, Shruti Asati[1], Bhavya Chiti[1]
[1]Department of Microbiology, Netaji Subhash Chandra Bose Medical College, Jabalpur, Madhya Pradesh, India
*Correspondence: Dr Riti Jain Seth (drsethriti@gmail.com)

ABSTRACT

Background: Biofilms are the assembly of bacterial species embedded in an extra-polymer matrix. Literature suggests biofilms can be formed on various biotic and abiotic surfaces. This may contribute to diseases by inducing chronic inflammation by underlying bacterial species. A prospective observational study was planned to isolate and identify bacterial pathogens among heterogenous clinical samples along with their antimicrobial resistance pattern and to study their tendency to form biofilm.

Materials and Methods: Samples were processed and identified according to standard microbiological protocol. Further Tissue Culture Plate (TCP) method was employed for detection of biofilm formation. Data analysis was done using descriptive statistics, Chi-square test & Fisher test.

Results: Among 329 positive bacterial isolates, 295 isolates were gram negative while only 35 isolates were gram positive. 138 (41.95%) bacterial isolates produced biofilm while 191 (58.05%) isolates were non-biofilm producers. Antibacterial resistance was higher in biofilm producing isolates as compared to non-biofilm producing isolates.

Conclusions: Timely information about biofilm producing bacterial species causing infection can help clinicians for appropriate treatment measures in addition to antibiotic therapy. Knowledge regarding these organisms could help us in formulating hospital antibiotic policy which will lead to better patient outcome.

Keywords: Biofilm, Bacterial infections, Antimicrobial resistance, Tissue Culture Plate method

INTRODUCTION

In most environmental niches, bacteria survive & multiply not as planktonic cells suspended in liquids but as surface-attached biofilms.[1] Biofilms are the most common survival mode of bacterial growth in nature as well as in clinical infections.[2] Biofilm associated diseases pose considerable diagnostic challenges for the clinical microbiological laboratory. These include false negative cultures, low colony count and decreased antimicrobial susceptibility. Various phenotypic and genotypic methods are available for assessing biofilm forming ability of microorganisms, but none of the methods is universally applicable because of inherent analytical limitations associated with measurements of bacterial adhesion. Some of these methods include Tissue Culture Plate method (TCP), Tube method, Congo red agar method, Flow cell method, Confocal laser scanning microscopy, Calgary biofilm device, ATP bioluminescent assay and molecular methods for identifying genes responsible for Extracellular Polymeric Substances (EPS) synthesis and bacterial adhesion. Out of the available methods, TCP is considered the gold standard phenotypic method of biofilm detection. In this method, bacterial adherence is measured spectrophotometrically. The optical density is measured after complete drying of tissue culture plate.[3-5]

As there are limited studies exploring biofilm formation and its clinical association from this part of the country, so, it was desirable to conduct a study involving heterogeneous patient population with the aim to study antibiotic susceptibility pattern and its association with biofilm

formation in bacterial isolates, thereby guiding the antibiotic therapy.

MATERIALS AND METHODS

This prospective study was conducted from April 2022 to September 2022 after due approval from institutional ethics committee. During the study period a total of 329 bacterial isolates from various clinical samples submitted in the laboratory were processed.

The samples submitted comprised of BAL, blood, pleural fluid, sputum, urine and pus samples isolated from different set of patients. Leaky, unlabelled inappropriately collected samples suggestive of contamination were excluded from the study. Also, duplicate samples collected from same patient were not included in the study. Specimens were processed following standard operating procedures for microscopy & culture of specific specimen. After culture, identification of the organism was done by colony morphology, gram staining and relevant biochemical reactions as per the protocol.[6] Antimicrobial susceptibility testing was done by modified Kirby-Bauer disc diffusion method according to CLSI guidelines 2022.[7] Biofilm production was detected by TCP method.[3,5] Brain Heart Infusion (BHI) broth was prepared & was supplemented by adding 2% sucrose; 0.5ml (500µL) of this broth was dispensed in labelled test tube & test organisms were inoculated and kept for overnight incubation at 37˚ C. On next day, these inoculated test tubes were diluted in the ratio of 1:100 by adding 5 ml of fresh BHI broth. A 200µL of this diluted culture broth was dispensed in 96 flat bottom wells, non-treated polystyrene tissue culture plate & was further incubated overnight at 37˚ C. Next day, the content of the wells were removed & wells were washed five times with phosphate buffer solution. Then wells were fixed with absolute alcohol by dispensing 200µL in each well for ten minutes and they were stained by adding 0.1% Crystal violet for thirty minutes. Excess stain was rinsed off after thirty minutes. After drying, optical density was determined by an automated ELISA reader at wavelength of 570 nm and classified into 3 categories as shown in Table-1.

Table-1: Classification of bacterial adherence by Tissue Culture Plate method[3]

Biofilm formation	Mean OD value
None/weak	< 0.120
Moderate	0.120 - 0.240
Strong	> 0.240

Statistical analysis: The presentation of the categorical variables was done in the form of number and percentage (%). The association of the variables which were qualitative in nature was analyzed using Chi-Square test. If any cell had an expected value of less than 5 then Fisher's exact test was used. For statistical significance, p value of less than 0.05 was considered statistically significant. The final analysis was done with the use of Statistical Package for Social Sciences (SPSS) software, IBM manufacturer, Chicago, USA, ver. 25.0.

RESULTS

A total of 329 clinical samples with positive bacterial isolates were included in the study, which consisted of pus samples from infected orthopedic implants, pus from wounds, blood, sputum, BAL, pleural fluid and urine. Among 329 positive bacterial isolates, 138 (41.95%) bacterial isolates produced biofilm while 191 (58.05%) isolates were non-biofilm producers.

Among biofilm producers, we observed significantly higher male preponderance with highest number of biofilm producers in age group of 15-45 years. In age group less than 15 years biofilm producers were least in number. Any particular type of source sample to have significantly higher tendency to form biofilm was not observed (Table-2).

Table-2: Biofilm producers/ non-biofilm producers in different specimen

Sample	Biofilm producers (n=138)	Non-biofilm producers (n=191)	P value
BAL	6 (60%)	4 (40%)	0.331*
Blood	33 (36.26%)	58 (63.74%)	0.197†
Pleural fluid	7 (58.33%)	5 (41.67%)	0.241†
Pus	60 (41.10%)	86 (58.90%)	0.78†
Sputum	14 (42.42%)	19 (57.58%)	0.953†
Urine	18 (48.65%)	19 (51.35%)	0.38†

* Fisher's exact test, † Chi square test

Among 329 bacterial isolates, 295 isolates were gram negative while only 35 isolates were gram positive. Among gram negative isolates, 118 (40%) isolates produced biofilm while among 35 gram positive isolates, 20 (58.82%) isolates produced biofilm with significantly higher tendency of gram positive bacterial isolates to produce biofilm (p-value 0.035). Among gram positive biofilm producers, Staphylococcus aureus had significantly higher (p-value 0.034) tendency to form biofilm followed by Coagulase negative Staphylococcus and Enterococcus species. Among gram negative biofilm producing isolates, Pseudomonas aeruginosa (57.32%) had maximum tendency to form biofilm followed by Klebsiella pneumoniae (37.72%) and Escherichia coli (28.28%) (Table-3).

In the present study, antibiotic resistance pattern in all bacterial isolates that included biofilm producers as well as non-producers were studied in detail. A particular set of antibiotics were used to study the resistance pattern in gram negative & gram-positive bacterial isolates in accordance

with our institutional protocol. Among gram negative biofilm producing isolates, we observed significantly higher resistance to drugs such as Ceftazidime (p-value 0.004), Gentamycin (p-value .0002), Amoxycillin-clavulanic acid (p-value 0.005), Imipenem (p-value 0.03), Netilmicin (p-value 0.015) & Doxycycline (p-value 0.006) as compared with non-biofilm producing gram negative isolates (Table-4).

Table-3: Association of organism isolated with biofilm producers/non biofilm producers

Organism isolated	Biofilm producers (n=138)	Non-biofilm producers (n=191)	P value
Staphylococcus aureus	15 (62.50%)	9 (37.50%)	0.034†
Coagulase negative staphylococcus	4 (57.14%)	3 (42.86%)	0.459*
Enterococcus spp.	1 (33.33%)	2 (66.67%)	1*
Escherichia coli	28 (28.28%)	71 (71.72%)	0.001†
Klebsiella pneumoniae	43 (37.72%)	71 (62.28%)	0.258†
Pseudomonas aeruginosa	47 (57.32%)	35 (42.68%)	0.001†

* Fisher's exact test, † Chi square test

Table-4: Association of antibiotics resistance pattern with biofilm producers/non biofilm producers in gram negative bacterial isolates

Antibiotics	Resistance among Biofilm producers	Resistance among Non-biofilm producers	P value
Ceftazidime (CAZ)	117 (100%)	164 (93.71%)	0.004*
Gentamicin (GEN)	82 (70.09%)	84 (48%)	0.0002†
Amoxycillin-clavulanic acid (AMC)	53 (75.71%)	79 (55.63%)	0.005†
Piperacillin-tazobactam (PTZ)	10 (8.55%)	19 (10.73%)	0.538†
Amikacin (AK)	62 (59.62%)	87 (50.58%)	0.145†
Cefepime (CPM)	50 (89.29%)	31 (77.50%)	0.117†
Ciprofloxacin (CIP)	77 (66.38%)	102 (57.63%)	0.133†
Meropenem (MER)	10 (8.55%)	9 (5.11%)	0.242†
Imipenem (IPM)	17 (14.53%)	12 (6.82%)	0.03†
Netilmicin (NET)	34 (70.83%)	16 (44.44%)	0.015†
Co-trimoxazole (COT)	43 (61.43%)	72 (51.06%)	0.155†
Doxycycline (DOX)	53 (76.81%)	78 (57.35%)	0.006†

* Fisher's exact test, † Chi square test

Among gram positive bacterial isolates, biofilm producing isolates exhibited significantly higher resistance to Amoxicillin-clavulanic acid (p-value .0008), Co-trimoxazole (p-value 0.0007), Doxycycline (p-value 0.0009), Erythromycin (p-value <0.0001), Cefoxitin (p-value 0.0002) and Penicillin (p-value 0.022). No resistance gram positive isolates to Vancomycin were observed in either of the groups (Table-5).

Table-5: Association of antibiotics resistance pattern with biofilm producers/non biofilm producers in gram positive

Antibiotics	Resistance among Biofilm producers	Resistance among Non-biofilm producers	P value
Amoxicillin-clavulanic acid (AMC)	15 (78.95%)	2 (15.38%)	0.0008*
Co-trimoxazole (COT)	18 (90%)	4 (30.77%)	0.0007*
Doxycycline (DOX)	14 (70%)	1 (7.69%)	0.0009*
Erythromycin (E)	17 (85%)	2 (14.29%)	<.0001*
Clindamycin (CD)	7 (35%)	2 (14.29%)	0.25*
Cefoxitin (CX)	17 (89.47%)	3 (21.43%)	0.0002*
Penicillin (P)	20 (100%)	10 (71.43%)	0.022*
Vancomycin (VA)	0 (0%)	0 (0%)	NA

* Fisher's exact test

DISCUSSION

The tendency of a bacterial isolate to develop biofilm is associated with the capacity of the organism to survive with in hospital environment. The frequency of the etiological agents varies among different published studies. Among overall gram-negative isolates, non-lactose fermenting gram negative organism Pseudomonas aeruginosa accounted for the most prevalent bacteria to form biofilm, followed by the gram-negative lactose fermenter bacteria Klebsiella pneumonia and E. coli. Present study shows remarkably low prevalence of gram-positive bacterial isolates as compared to gram negative isolates which may be attributed to different subset of patients more prone to gram negative bacterial infections such as urinary tract infection[8], infected implants & catheters[9], infected wounds[10] and hospital acquired pneumonia etc. This shift in prevalence towards gram negative bacteria in this study may also be attributed to the routine antibiotic prophylaxis[11] and sterile skin preparation protocol[12] followed prior to any invasive procedure.

Gram positive isolates showed higher tendency to form biofilm which was also observed by Sarangi et al.[13] Among gram negative isolates, Pseudomonas aeruginosa, Klebsiella pneumonia and E. coli were found to be most prevalent

biofilm producers. Similar observations were also found by Harika et al.[10]

In our study, we observed significantly higher level of resistance to the commonly prescribed drugs at our institution such as penicillins, cephalosporins, fluoroquinolones, aminoglycosides and tetracyclines with the biofilm producing bacterial isolates as compared to non-biofilm producing bacterial isolates. The resistance to carbapenems such as Meropenem, Imipenem was also found to be more prevalent among biofilm producing gram negative isolates. But gram-positive isolates didn't show any resistance to Vancomycin. In a study by Dumaru et al[14] similar resistance pattern among biofilm producing gram negative isolates was observed. Comparable resistance pattern was also observed by Cepas et al[15] and Asati et al[3] in biofilm producing gram negative isolates. Resistance among biofilm producing isolates were also found to be prevalent to commonly used antibiotics in a study conducted by Harika etal.[10]

We found biofilm formation high in methicillin resistant Staphylococcus aureus (MRSA) strains, further confirming biofilm formation results in enhanced overall resistance. Similar findings were noted by Vanessa et al.[16] Mehta et al also observed high resistance to aminoglycosides, fluroquinolones and cephalosporins by gram-negative bacterial isolates and netilmicin & erythromycin by gram-positive bacteria in biofilm producers in burn wound infections.[17]

Our observation pattern of high antimicrobial resistance may be attributed to the fact that ours is a tertiary care center, hence we get so many referrals from primary centers, where patients are already on ingenious use of antibiotics. Injudicious use of antibiotics also leads to selection pressure that may favor the acquisition of resistance among micro-organisms including biofilm formation.

CONCLUSIONS

This study emphasize that microorganisms have a tendency to develop biofilm on various clinical sites. This process of formation of biofilm is associated with increased resistance to antimicrobial agents. This might be due to the fact that they act as a persistent source of infection. However, routine antimicrobials administration is not sufficient to treat such infections due to poor drug penetration. Therefore, methods to prevent their formation as well as for their removal should be developed. Also, there is a need to routinely detect biofilm producing strains. This would guide healthcare providers to develop effective patient management strategies.

Limitation of the study
Molecular methods identifying gene responsible for biofilm production were not included due to lack of facility.

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