Comparision of Three Different Methods for the Detection of Biofilm in Gram Positive Cocci and Gram Negative Bacilli Isolated from Clinical Specimens

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Abstract:
Background:
Biofilm production is an important virulence factor leading to chronicity of many infections. It is also responsible for the emergence of multidrug resistant strains resulting in treatment failure.
Aim of the study:
To detect biofilm production in clinical isolates by microtitre plate method, Tube adherence method, Congo red agar method and to evaluate sensitivity and specificity of these methods. To compare these three methods to know which is the most efficient method of biofilm detection. To know the antibiotic resistance pattern of biofilm producing isolates.
Material and *Methods:*
A total of 70 clinical isolates were tested for biofilm production by microtitre plate method, tube adherence method and congo red agar method. All the isolates were subjected to antimicrobial susceptibility testing by Kirby Bauer Disk Diffusion method as per CLSI guidelines.
Results:
Out of 70 isolates, 25 were gram positive cocci and 45 were gram negative bacilli. Biofilm production was seen in 19 (76%) of the 25 gram positive cocci and 42 (93%) of the 45 gram negative bacilli. Microtitre plate method was the most effective method and detected biofilm production in 76% of gram positive cocci and in 93% of gram negative bacilli. Sensitivity and specificity of microtitre plate, tube and congo red agar methods were 98.08%, 71.88%, 12.7% and 61.22%, 88.89%, 86.2% respectively. Maximum resistance in biofilm producing gram positive isolates was seen to Penicillin (100%) and Cephalexin (100%) while in biofilm producing gram negative isolates maximum resistance was seen to Ampicillin (83.78%) and Ciprofloxacin (70.27%).
Conclusion:
 Microtitre plate method was found to be the most efficient method with sensitivity and specificity of 98.08% and 61.22% respectively. Microtitre plate method is a quantitative and reliable method and can be recommended as a screening method. Detection of biofilm production helps in better clinical management.
Keywords: Biofilm, Microtitre Plate method, Tube adherence method, Congo red agar method.

INTRODUCTION:

Biofilm is a microbial community which is embedded in extracellular matrix [1, 2]. They are colonial way of life of microorganisms and defined as complex microbial assemblages anchored to abiotic or biotic surfaces such as plastic, metal, glass, soil particles, wood, medical implant materials, tissue and food products [3]. Attachment is by fimbriae, pilli, flagella, extracellular polymeric substance which acts as a bridge between bacteria and the conditioning film [4]. The cells which are embedded in extracellular matrix interact with the environment and with each other by the chemotactic particles or pheromones called as quorum sensing [5, 6]. This miniature ecosystem provides safe home for the members of the community which are unaffected by the defence mechanisms of host immune responses, phagocytosis and antibiotic treatment [7, 8]. The ability of a microorganism to develop biofilm is an important virulence factor and they are the main cause of many chronic infections. They are responsible for the emergence of multidrug resistant strains resulting in treatment failure [9]. For better clinical management especially chronic infections, it is necessary to detect production of biofilm. Various methods have been devised for the detection like microtitre plate method, tube adherence method, congo red agar method,

spectrophotometry and transmission electron microscopy. Both gram positive and gram negative bacteria have the ability to form biofilms. Bacteria commonly involved include *E. faecalis, S. aureus, S. epidermidis, E. coli, K. pneumoniae, P. mirabilis and P. aeruginosa.*
With this background, the present study was undertaken with the aim to detect the production of biofilm among the clinical isolates by using three different methods : microtitre plate method, tube adherence method, congo red agar method ; evaluate the sensitivity and specificity in order to determine the most suitable screening method for biofilm detection and to know the antibiotic resistance pattern among biofilm producing isolates.

MATERIAL AND METHODS:

Source of data: Seventy isolates from various clinical specimens obtained from patients attending BLDEA'S Shri B.M. Patil Medical College Hospital and Research Centre, Vijayapur.
Study design: A prospective study conducted from January 2014 to June 2014.
Sampling : According to Mathur T *et al.* study, detection of biofilm by microtitre plate method, tube method and congo red agar methods were 39%, 30% and 4% respectively [14]. Considering average detection of biofilm formation 24%, α level 0.05 & 0.05 desired precision of

estimate, the sample size is 70 using the formula ,

$$n = \frac{z^2 \times p \times (1-p)}{e^2}$$

z = desired confidence level 95%
p = proportion value
e = desired precision

Methods: A total of 70 isolates of gram positive cocci and gram negative bacilli from various clinical specimens were subjected to biofilm detection by three different methods - microtitre plate method (MTP), tube adherence method (TM) and congo red agar method (CRA). Antibiotic susceptibility testing was done by Kirby Bauer Disk Diffusion method.

Microtitre plate method:
Twenty microlitres of overnight bacterial culture was added in sterile 96 well flat bottomed polystyrene microtitre plates containing 230 µl of trypticase soy broth. The plates were incubated aerobically at 35° C for 24 hrs. The content of the wells were poured off and washed 3 times with 300 µl of sterile distilled water. The bacteria adhering to the wells were fixed with 250 µl of methanol for 15 min. Then the wells were washed with sterile distilled water, followed by staining with 250 µl of 1% crystal violet solution for 5 min. Excess stain was removed by washing and air dried. The dye bound to the wells was resolubilised with 250 µl of 33% glacial acetic acid. Then the optical density of each well was measured at 490 nm using an ELISA reader [10]. Cut off OD was determined as three standard deviations above the mean OD of the negative control.

Tube adherence method:
About 10 ml of trypticase soy broth with 1% glucose was inoculated with a loopful of microorganisms from overnight culture plates and incubated at 37°C for 24 hrs. The tubes were decanted and washed with phosphate buffered saline and dried. Dried tubes were stained with 0.1% crystal violet. Tubes were washed with deionized water. Slime formation was considered positive when a visible film lined the wall and bottom of the tube. Ring formation at the interface was not considered of slime production [11].

Congo red agar method:
Congo red agar medium consists of brain heart infusion 37 gm/l, sucrose 50 gm/l, agar 10 gm/l and congo red stain 0.8 gm/l. Congo red stain was prepared as a concentrated aqueous solution and autoclaved at 121° C for 15 min separately and added to the growth medium cooled to 55° C. The test isolates were inoculated on to the CRA plates and incubated aerobically at 37°C for 24 hrs. Production of black colonies with a dry crystalline consistency was considered as a positive result. Weak slime producers were indicated by pink colonies with occasional darkening at the centre of colonies. Non slime producing organisms produced pink colonies [12].
Antibiotic sensitivity testing was done by Kirby Bauer disk diffusion method as per CLSI guidelines [13].

Statistical analysis:
Statistical analysis of the data was done by applying McNemer's chisquare test and sensitivity and specificity was calculated.

RESULTS:
Out of 70 isolates, 30 were from pus, 22 from urine and 18 from ascitic fluid, pleural fluid, sputum and ear discharge. Of the 70 isolates, 25 were Gram positive cocci and 45 were Gram negative bacilli. Most common isolate was *S.aureus* (44%) amongst gram positive cocci and *E.coli* (44.44%) amongst gram negative bacilli. Microtitre plate method is the gold standard method followed. Biofilm production was seen in 19 (76%) of the 25 gram positive cocci and microtitre plate method was found to be the most effective method with the detection of biofilm production in 76% of gram positive cocci (Table 1). Association of biofilm detection in gram positive cocci by microtitre plate method was statistically significant (p value 0.05).

Table 1: Detection of biofilm in gram positive cocci by MTP, TM, CRA

Organism	Total	MTP		TM		CRA	
		P	N	P	N	P	N
S.aureus	11	10	1	7	4	3	8
Enterococcus spp	7	5	2	2	5	2	5
CONS	2	1	1	1	1	0	2
S.pyogenes	3	3	0	2	1	1	2
S.pneumoniae	2	0	2	0	2	0	2
p value		0.05		0.36		0.81	

(P-Positive, N-Negative, MTP-Microtitre plate method, TM-Tube adherence method, CRA-Congo red agar method)

Biofilm production was seen in 42 (93%) of the 45 gram negative bacilli and microtitre plate method was the most effective method with the detection of biofilm production in 93% of gram negative bacilli (Table 2).

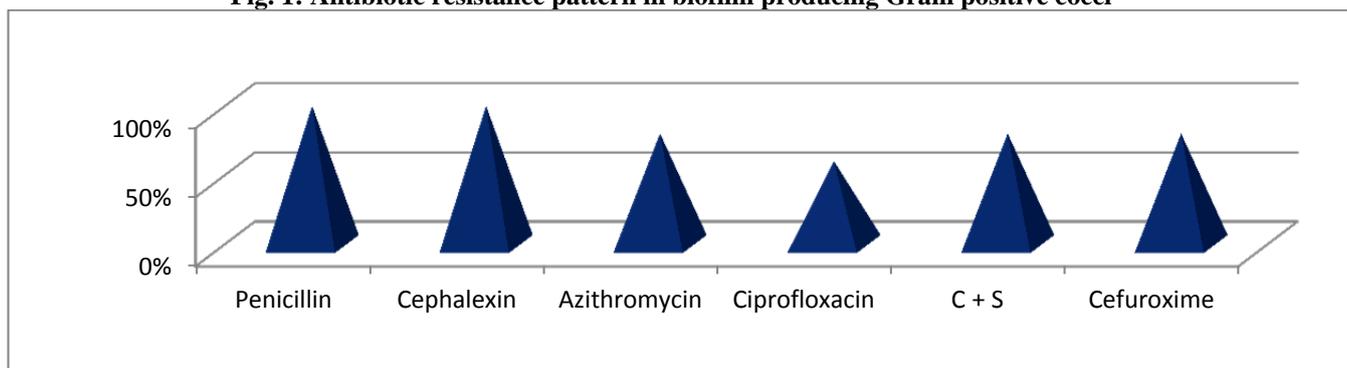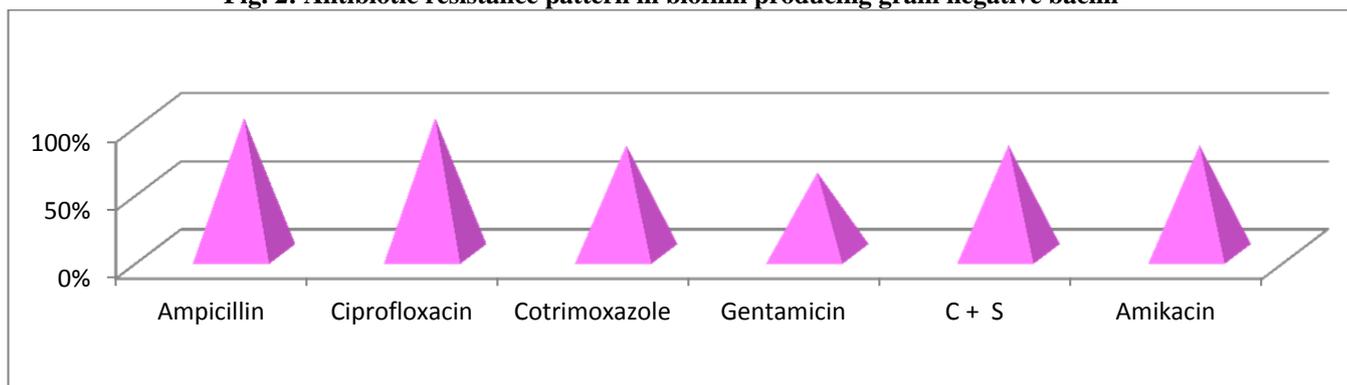**Table 2: Biofilm detection in Gram negative bacilli by MTP, TM, CRA**

Organism	Total	MTP		TM		CRA	
		P	N	P	N	P	N
E.coli	20	19	1	14	6	10	10
P.aeruginosa	12	12	0	4	8	5	7
K.pneumoniae	10	8	2	6	4	3	7
Citrobacter spp	3	3	0	2	1	1	2
p value		0.25		0.231		0.75	

(P- Positive, N- Negative, MTP-Microtitre plate method, TM-Tube adherence method, CRA-Congo red agar method)

When compared to other methods, microtitre plate method was found to be the most effective method in the detection of biofilm production with the sensitivity and specificity of 98.08% and 61.22% respectively (Table 3).

Table 3: Sensitivity and specificity of the three methods

Method	Sensitivity	Specificity
Microtitre plate	98.08%	61.22%
Tube adherence	71.8%	88.8%
Congo red agar	12.7%	86.2%

Fig. 1: Antibiotic resistance pattern in biofilm producing Gram positive cocci

Fig. 2: Antibiotic resistance pattern in biofilm producing gram negative bacilli

Antibiotic resistance among biofilm producing gram positive isolates was tested for Penicillin (10 μg), Cephalexin (30 μg), Azithromycin (15 μg), Ciprofloxacin (5 μg), Cefoperazone + Sulbactum (C+S) (30 μg) and Cefuroxime (30 μg). Maximum antibiotic resistance in biofilm producing isolates was seen to Penicillin (100%) and Cephalexin (100%). Maximum antibiotic resistance in non biofilm producing gram positive cocci was seen to Penicillin (66.66%) and Azithromycin (33.33%). Figure 1 shows antibiotic resistance pattern in biofilm producing Gram positive cocci.

Antibiotic resistance was tested in gram negative bacilli for Ampicillin (10 μg), Ciprofloxacin (5 μg), Cotrimoxazole (25 μg), Gentamicin (10 μg), Cefoperazone + Sulbactum (C+S) (30 μg) and Amikacin (30 μg). Maximum resistance in biofilm producing gram negative bacilli was seen to Ampicillin (83.78%) and Ciprofloxacin (83.78 %). Maximum antibiotic resistance among non biofilm producing gram negative bacilli was seen to Ampicillin (33.33%) and Ciprofloxacin (33.33%). Figure 2 shows antibiotic resistance pattern in gram negative bacilli

DISCUSSION:

In the present study biofilm production was detected in 91% of gram negative bacilli which is nearly similar to Zubair *et al* and Swarna *et al* studies which showed 80% and 91% of biofilm detection in gram negative bacilli respectively. Our study showed biofilm production in 88 % of gram positive cocci which is higher than that observed by Swarna *et al* (47%). Maximum biofilm production in *P. aeruginosa* (100%) amongst gram negative bacilli was come across in our study which is not in accordance with Zubair *et al* study which reported *P.vulgaris* (80%).

S.aureus (90%) was the predominant gram positive cocci which showed maximum biofilm production which is in correlation with Swarna S *et al* study [7,9]. Present study reported maximum biofilm production in isolates from pus while in a study by Hassan *et al* maximum biofilm producing isolates were from urine (30%) [5].

Sensitivity and specificity of 98.08% and 61.22% in microtitre plate method was observed in our study with sensitivity being in accordance with Mathur T *et al* & Oliviera *et al* (97.1% and 97.6%) and specificity is lower than that observed in Mathur T *et al* & Oliviera *et al* (97.5% and 94.4%) [14,12]. Sensitivity and specificity of tube method was found to be 71.88% and 88.89% in the present study which is similar to Hassan *et al* study with sensitivity of 73% and specificity of 92%. We reported sensitivity and specificity of 12.7% and 86.2% for congo red agar method which is in correlation with the specificity of Mathur *et al* study (90.02%) while sensitivity is observed higher than that [14]. Gold standard method followed by these methods was Microtitre plate method.

We observed maximum resistance in biofilm producing gram positive isolates to Penicillin (100%) and Cephalexin (100%) which is similar to Hassan *et al* and Sasirekha *et al* studies which also showed maximum resistance to Penicillin (100%) [5,13]. In another study by Zubair *et al* maximum resistance was seen to Cefoperazone [7].

Maximum resistance in biofilm producing gram negative isolates to Ampicillin (83.78%) and Ciprofloxacin (70.27%) was detected by us that are also similar to Hassan *et al* study while Murugan *et al* and Zubair *et al* studies have reported maximum resistance to Amikacin (71.9%) and Cefoperazone (79.6%)[5,15,7].

CONCLUSION:

Biofilm production leads to chronicity of infections and its infectious complications. It is responsible for persistence of organisms in infection sites and hospital environment. It is an important barrier to effective treatment. So biofilm detection helps in better clinical management. In the present study, biofilm production was detected maximum in gram negative bacilli (91%). Microtitre plate method is found to be the most efficient method with sensitivity and specificity of 98.08% and 61.22% respectively. It is a quantitative and reliable method and can be recommended as a screening method. Maximum antibiotic resistance was seen among biofilm producing isolates.

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