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INVITRO DETECTION OF BIOFILM FORMATION IN CLINICAL ISOLATES AND COMPARISON WITH ANTIBIOGRAM

Microbiology

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ABSTRACT

Background: Biofilms cause increased antimicrobial resistance and persistent infection. Aim:To detect biofilm production in clinical isolates using phenotypic tests and study antibiogram. **Method:** A prospective cross sectional study conducted in Microbiology , KMCH IHSR. Total 94 isolates identified by standard procedures were subjected to biofilm detection by Tube & Tissue Culture plate method. Antibiogram was performed by disc diffusion. **Results:** The most common organism was Escherichia coli (42%) followed by Klebsiella species (19%). Predominant Gram-positives, were Staphylococcus aureus and Enterococcus species (36%). 6 isolates were completely resistant to antibiotics. TCP was superior to tube method. The total number of biofilm production by tube and TCP method was 36(38.3%), only TCP method 46 (49%) and either by tube method or TCP method 82 (87%). Biofilm producers showed higher resistance. **Conclusion:** Simple, cost-effective test may be conductedroutinely to assist surgeons / clinicians in effectively managing infections, reducing mortality and morbidity.

KEYWORDS

INTRODUCTION:

Biofilms are a community of microbes attached to a substratum and enclosed in a substance called extracellular polymeric substance (EPS) produced by the encased microbial cells (1). The term "**biofilm**" was formally introduced in 1978 by Costerton (2). Biofilms are a serious threat for public due to the increased capacity of resistance seen in the biofilm-encased organism to antimicrobials and the persistence of infection (3). As per NIH (National Institute of Health), infections caused by biofilm producing organisms accounts to about **65-80%**. Bacterial biofilm has variable morphologies. Bacteria form biofilm as a survival strategy enabling them to survive in unpredictable stressors from the environment such as temperature, desiccation, UV and pressures (4). The bacteria that have the capability to form biofilms are phenotypically different from the ones that do not form biofilm. The physiological state of the bacteria changes making them more resistant to antibiotics(5).

Numerous methods like tube method (TM), tissue culture plate method (TCPM), Congo-red agar method (CRA), confocal scanning microscopy, electron microscopy, and bioluminescent assay are the phenotypic detection methods available for the identification of biofilm formation. Genotypic detection by targeting genes involved in biofilm formation using PCR has been extensively used, but this is inconvenient to be used routinely for diagnostic purposes due to its expensiveness (6,7). Therefore, in the present study, we used phenotypic assays such as the tissue culture plate method (TCPM) and tube method (TM) for biofilm identification.

In the present study, we have intended to determine the prevalence of biofilm producers and correlate the result of the antimicrobial susceptibility testing. The information of biofilm production to the clinicians will guide them in using this knowledge to decide on therapy that improves patients health and well-being.

METHODOLOGY:

Our study is a cross-sectional prospective study that was conducted in the Department of Microbiology, KMCH IHSR, after obtaining approval from the Ethics Committee. The study included 94 nonrepetitive, consecutive bacterial isolates from clinical samples like urine, pus, sputum, wound swab, etc. over 6months. All the above samples were plated onto sheep Blood Agar Plates (SBAP)(5%) and MacConkey Agar Plates (MAC) and allowed to grow aerobically at 37 degrees C for 18-24 hours. Pure isolated colonies of organisms were further identified using biochemical tests, and antimicrobial susceptibility testing using the Kirby Bauer disc diffusion method following the guidelines of the clinical and laboratory standards institute (CLSI, 2021) (8). All the materials required for the isolation, identification and antibiogram were obtained from HiMedia Laboratories India. Quality control of antibiogram: All the antibiotic discs were tested against Pseudomonas aeruginosa ATCC 27853 , Staphylococcus aureus ATCC 25923 and Escherichia coli ATCC 25922 to ensure their potency (8).

The antibiotics used for testing gram positive bacteria were penicillin (10 units), ampicillin (10 µg), cefoxitin (30 µg), erythromycin (15 µg), vancomycin (30 µg), clindamycin (2 µglinezolid (30 µg), gentamicin (10 µg), high level gentamicin (120 µg), ciprofloxacin (5 µg), levofloxacin (5 µg), chloramphenicol (30 µg), tetracycline (30 µg), fosfomycin (200 µg), rifampicin (5 µg).

The antibiotics for gram negative bacteria were cefazolin (30 µg), ampicillin (10 µg), cefuroxime (30 µg), gentamicin (10 µg), amikacin (30 µg), ciprofloxacin 5 (µg), tetracycline (30 µg), trimethoprim-sulphamethoxazole (1.25/23.75 µg) cefotaxime (30 µg), cefotaxime+ clav (30/10 µg), ceftazidime (30 µg), cefoxitin (30 µg), cefepime (30 µg), cefazolin (30 µg) cefuroxime (30 µg), norfloxacin (10 µg), piperacillin (100 µg), amoxicillin clavulanic acid (20/10 µg), piperacillin tazobactam (100/10 µg), ceftriaxone (30 µg), ertapenem (10 µg), imipenem (10 µg), meropenem (10 µg), nitrofurantoin (300 µg), aztreonam (30 µg), tobramycin (10 µg), levofloxacin (5 µg), chloramphenicol (30 µg), doxycycline (30 µg), tetracycline (30 µg), tigecycline (15 µg), polymyxin B (50 units).

All bacterial isolates were then identified for biofilm formation by qualitative (TM) and quantitative (TCP) methodologies as described by Christensen *et al*.(9). The tissue culture plate results will be read in the form of optical density. Data were obtained and the results were analyzed using the Microsoft Excel and IBM SPSS statistics version 21.0 software (SPSS Inc., Chicago II, USA). The sensitivity, specificity, and predictive values were calculated.

RESULTS

The total samples received for culture in bacteriology during the study period was 43. Out of the 43 Samples, 23 were urine, 11 pus, 2 ear swabs, 2 blood, 2 fluids, 2 sputa, and 1 vaginal swab [Fig1]. From these 43 samples, 50 clinical isolates were obtained. Out of that, 14 (28%) were Gram-positive and 36 (72%) Gram-negative[Fig2].

The commonest organism isolated was *Escherichia coli* (42%) followed by *Klebsiella species* (19%) [Fig4]. Among the Gram-positive organisms, *Staphylococcus aureus* and *Enterococcus species* were predominant (36%). [Fig3]

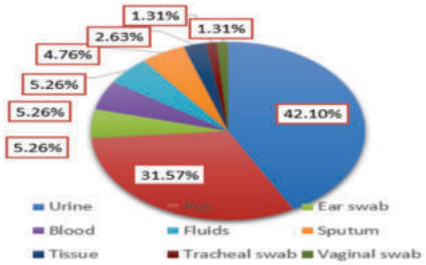

Fig.1 Profile of total clinical samples included in the study

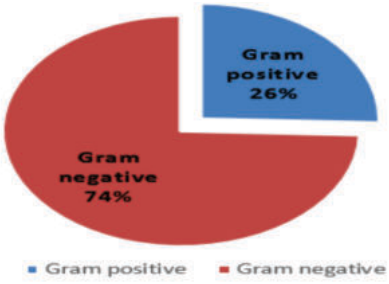

Fig.2 Distribution of microorganisms isolated from various clinical samples

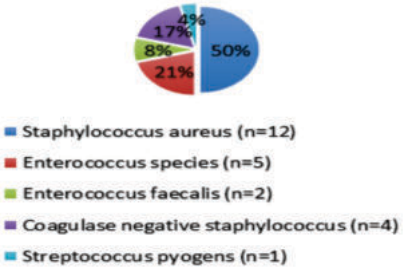

Fig.3 Profile of gram-positive organism isolated from clinical samples

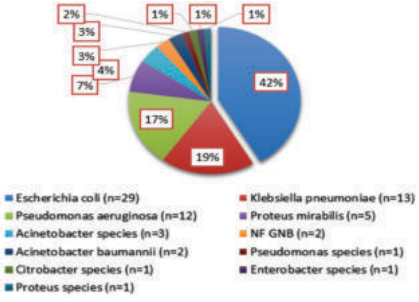

Fig.4 Profile of gram-negative organisms isolated from clinical samples

Antibiotic Susceptibility testing:

Among the total 50 isolates, 5 were completely resistant to all antibiotics, which were *Escherichia coli* (n=1), *Klebsiella pneumonia* (n=2), NFGNB (n=1), and *Enterobacter species* (n=1). The highest resistance was noted for ciprofloxacin (36%) by both gram-positive and negative organisms. Most of the gram positives were resistant to penicillin.

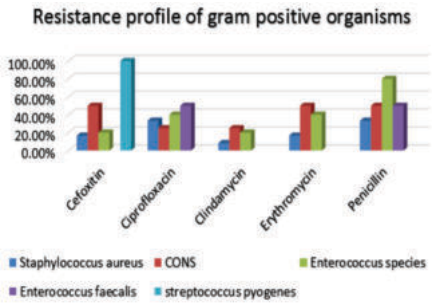

Fig. 5 Antibiotic resistance pattern of Gram-positive organisms.

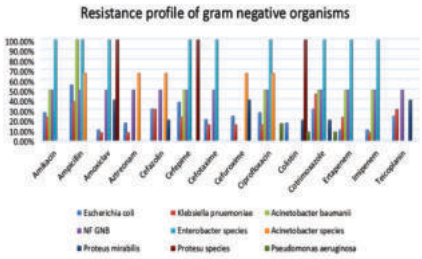

Fig.6 Antibiotic resistance pattern of Gram-negative organisms

Biofilm Detection:

A total of 50 isolates were studied for biofilm production using ATCC 27853 Pseudomonas aeruginosa as the positive control. The results of the QC (quality control) strain were satisfactory. The total number of isolates that showed biofilm formation by both the tube method and plate method was 17 (34%) [Fig7&8]. The number of isolates that showed biofilm production by TCP method alone but not by tube method is 26 (52%). Hence, the total isolates that showed biofilm production by either tube method or TCP method is 43 (86%) out of 50 (Table 1 & 2). There were no isolates that showed a positive result for biofilm detection by tube method only. Statistical analysis was done using a 2 × 2 table taking into account TCP as the gold standard method. The sensitivity, specificity, PPV (positive predictive value) and NPV (negative predictive value) of the tube method was 39.5%, 100%, 100%, and 21.2% respectively. The profile of biofilm producers among different samples is shown in fig 9

Table 1: Biofilm Detection By Different Methods

METHODS	BIOFILM PRODUCTION				NO BIOFILM PRODUCTION (%)
	Weak Positive	Moderate Positive	Strong Positive	Total Positive (%)	
Tube method	22	14	0	36(38.3)	58 (62)
Tissue culture plate method	24	23	35	82(87)	12(13)

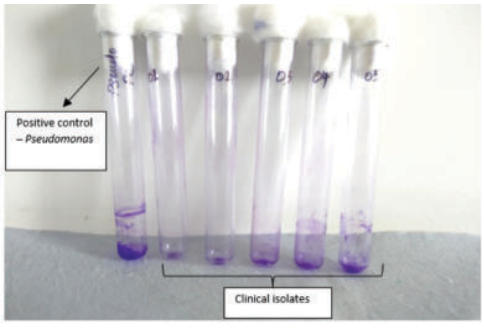

Fig. 7 Biofilm detection by tube method: First tube showing Positive control *Pseudomonas aeruginosa* ATCC 27853, Tubes 1 & 2 are biofilm negative clinical isolates, Tube 3 to 5 biofilm positive clinical isolates

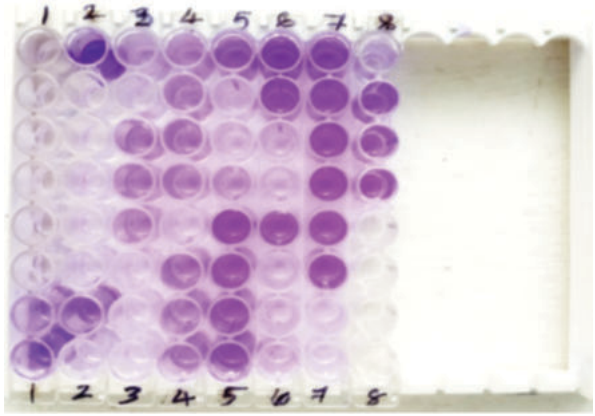

Fig. 8 Biofilm detection by tissue culture plate method: In strip 1 -A to C- Blank, D to F Negative control, G, H and strip 2 (A) – Positive control. Others are clinical isolates in triplets.

Table 2: Percentage Of Biofilm Production By Various Isolates

ORGANISM	PERCENTAGE OF BIOFILM FORMATION (%)		
	Tube Method	Tissue culture plate method	Both
Gram positive			
1. Staphylococcus aureus	2(16.6)	12 (100)	2(16.6)
2. Enterococcus species	0 (0)	4 (80)	0 (0)
3. Enterococcus faecalis	0 (0)	2 (100)	0 (0)
4. Coagulase negative staphylococcus	1(25)	3(75)	1 (25)
5.Streptococcus pyogenes	1(100)	1(100)	1(100)
Gram negative			
1. Escherichia coli	10(26.6)	22(73.3)	10(26.6)
2. Klebsiella pneumoniae	6(42.8)	11(85.7)	6(42.8)
3. Pseudomonas aeruginosa	10(83.3)	12(100)	10(83.3)
4. Proteus mirabilis	1(20)	4(80)	1(20)
5. Acinetobacter species	2(66.6)	3(100)	2(66.6)
6. Acinetobacter baumanii	0 (0)	2 (100)	0 (0)
7. NF GNB	1(50)	2(100)	1(50)
8. Enterobacter species	1(100)	1(100)	1(100)
9. Citrobacter species	0 (0)	1(100)	0 (0)
10. Pseudomonas species	1(100)	1(100)	1(100)
11. Proteus species	0 (0)	1(100)	0 (0)

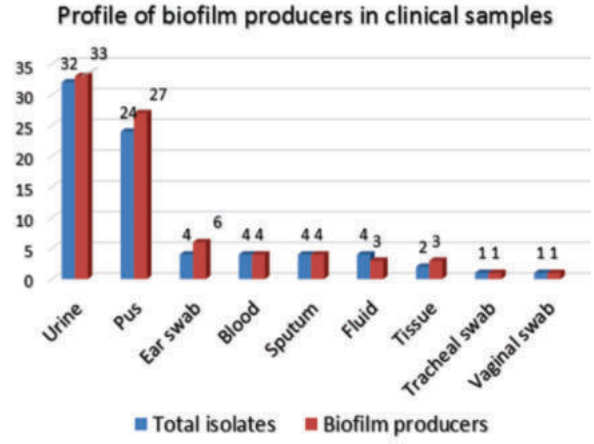

Fig. 9 Profile of biofilm producers among clinical samples

The antimicrobial susceptibility of the biofilm-forming isolates was also tested. Biofilm-producing strains seemed to show higher resistance than the biofilm non-producing isolates. 37.2% of biofilm producers were resistant to ciprofloxacin while the percentage for biofilm non-formers was 28.5%. An identical type of resistance pattern was also seen with some of the other antimicrobials used. (Fig. 10)

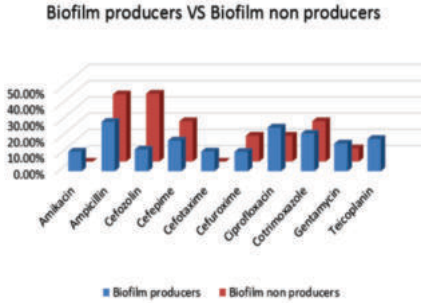

Fig. 10 Antibiotic resistance profile of biofilm producers vs biofilm nonproducers

DISCUSSION

Biofilm generated by the bacteria are mainly associated with persistence of bacteria in various environmental conditions for a long period of time. They are notoriously difficult to eradicate. Bacteria in biofilm exhibits exponentially increased resistance to antimicrobial agents. Antibiotic resistance takes several forms, including reduced antibiotic penetration into biofilms, decreased development, and the creation of resistance genes. Detecting biofilms can be done in a variety of methods (10)

In the present study, we collected 50 clinical strains from various samples to detect biofilm production and to compare the results with an antibiogram. Most isolates were obtained from urine samples from cases of suspected urinary tract infections (UTI) (Fig.1). Among the 50 isolates,36 were gram-negative and 14 were gram-positive. E. coli was the most prevalent pathogen with the occurrence of 42%. Similar findings were reported in several other studies that E.coli is the most commonly isolated pathogen in clinical isolates causing UTI, septicemia, and other infections 96. The most common gram-positive bacteria obtained in our study were Staphylococcus aureus and Enterococcus species (36%).

Analysis of the antibiogram of the clinical strains showed that all gram-positive and gram-negative organisms showed the highest resistance to ciprofloxacin (36%) (Fig 6&7). In the study done by Rani et al, the gram-negative organisms were highly resistant to cotrimoxazole 53% and ciprofloxacin 47% (11). The higher rate of resistance in gram-positive organisms towards penicillin is 45%. This is identical to the study by Banu et al (12)

In this study, we tested 50 isolates with two screening test methods (The tube method and the Tissue culture plate method) for their strength to form a biofilm (Fig7&8). The biofilm production was graded in both methods as strong, moderate, and weak. Overall, 86% of isolates were biofilm producers in our study which was greater compared to other studies in which it ranged from 73% to 77% (13)

The tube method showed 17 (34%) isolates were biofilm formers, was split into 9 (53%) moderate, 8 (47%) weak and 33 (66%) isolates were biofilm nonproducers. By tissue culture method 43 (86%) isolates were biofilm producers were 19 (44.2%) strong, 10 (23.25%) moderate, 14 (32.5%) weak and 7 (14%) isolates were biofilm nonproducers (Table3). In a similar study done by Rizal et al, out of 50, 32 were biofilm producers and 18 were none or weak biofilm producers. Among the biofilm producers, the most resistant antibiotic was cefotaxime in 20 and ceftriaxone in 16 and amoxiclav in 13, whereas amikacin was least resistant in 2 of the isolates (14). Study done by Saroj et al. showed 69% of isolates as biofilm producers. A study conducted by Sharma et al. showed production of bio film in 67.5% isolates of Escherichia coli(15)

The sensitivity, specificity,positive predictive value, and negative predictive value of the tube method by using tissue culture plate method as a gold standard were 39.5%,100%, 100%, and 22.1% respectively. The specificity and sensitivity of the present study showed almost similar findings as many other studies (sensitivity= 61% to 100%, Specificity= 66% to 100%)(10,16). The detection of biofilm by tube method was cumbersome to differentiate between the strength of biofilm formation as moderate, weak, and absent. There was a lot of inter-observer variation noted while interpreting the test results. As evident in the preceding studies, the tube method is not good enough to be considered as a general screening test to detect biofilm-producing isolates (10).

Biofilm detection among gram-positive organisms (Table 4), was an 85.7% positive result shown by the tissue culture plate method and 14.3% by tube method. By tissue culture plate method strong biofilm formation was seen in Staphylococcus aureus (40%). Many existing literature has supported the the biofilm-forming nature of Staphylococci (12). Among the 5 Enterococcus species, 4(80%) isolates produced biofilm only in the tissue culture plate method, 60% were moderate and 20% were weak. Enterococcus faecalis showed 100% weak biofilm by the tissue culture plate method. In the study by Shridhar et al, among the 137 Enterococcus tested for biofilm production, 2 Enterococcus species and 3 Enterococcus faecalis were strong biofilm producers (17). While comparing this result with our study result shows a high-level margin difference because of the number of isolates. CONS produced 50% moderate biofilm by tube method, tissue culture plate method. Biofilm detection in gram-negative organisms (Table 2) was 86.1% biofilm production shown by tissue culture plate method and 41.7% by tube method. Strong biofilm production was seen in Pseudomonas aeruginosa (83.3%), Acinetobacter baumanni, Acinetobacter species, Proteus mirabilis, and NFGNB (100%). Percentage of biofilm producers by Pseudomonas aeruginosa, Acinetobacter baumanni, Acinetobacter species, Proteus mirabilis, and NFGNB shows higher than data observed by Lima et al, Perez et al where 75% of the clinical isolates of Pseudomonas aeruginosa were biofilm producers (18). Srinivasa et al showed 62% of biofilm production in Acinetobacter baumanni (19).

Escherichia coli produced 73.3% biofilm by tissue culture plate method among which 26.6% were weak, 20% were moderate and 26.7% were strong. By tube method 26.6% Escherichia coli produced biofilm of which 13.3% were weak and moderate. The prevalence of biofilm-producing Escherichia coli in Dumeru et al studies was 63%. Klebsiella pneumonia showed 85.7% biofilm production by tissue culture plate method were 42.8% weak, 14.3% moderate, 28.6% strong and 42.8% and 28.5% weak, and 14.3% moderate by tube method.77.55% of Klebsiella pneumonia produced biofilm in the study conducted by Dumaru et al (20). In the present study, 100% of biofilm production was shown by Citrobacter species, Acinetobacter Species, and Acinetobacter baumanni by tissue culture plate method. Proteus mirabilis and NFGNB showed 100 % biofilm production by tissue culture plate method but only 50% by tube method (moderate). On the contrary, there were no biofilm-producing isolates of Acinetobacter species and Proteus mirabilis found in the study done by Tayal et al (21).

While seeing biofilm production by both methods Enterobacter species was the highest biofilm producer (100%) followed by Pseudomonas aeruginosa (83.3%), CONS, and Proteus mirabilis (50%), Klebsiella pneumonia (42.8%) (Table 2). Jose Ramos et al found that 16% of Escherichia coli strains, 73% of Klebsiella pneumonia strains, and 4% of Enterobacter strains revealed either moderate or strong biofilm production (22). Percentage of biofilm production by CONS, Klebsiella pneumonia are similar to the study conducted by Abdallah et al, where highest predominance was detected in CONS 57% (23).

The isolates from the urine and pus samples had more biofilm production in our study. The maximum sample that was received was urine the higher biofilm formation is also in the urine sample. Other studies done by Hassan et al showed that 30% urine, 25.7% urinary catheter tips, 12.8% pus, 11.4% Sputum, 10% intravenous catheter tips, and 10% naso bronchial lavage specimen isolates were biofilm producers from the clinical samples (10) (Fig 9).

The antibiogram was also studied based on the biofilm-forming ability of the isolates (Fig10). Nonbiofilm formers were the predominant bacterial isolates. But in bacterial isolates where there were multi-drug resistance (MDR) features, higher numbers of biofilm formers were predominant. In isolates with resistance to five or more antimicrobial agents, strong biofilm formers (86%) were seen.

In a study done by Poovendran et al., all biofilm producing bacteria were maximum resistant to amoxiclav (100%), ceftazidime (84%), piperacillin with tazobactam (83%) ,gentamicin and cefotaxime (86% each), chloramphenicol (100%), cotrimoxazole, and amikacin (70%). Resistance to co-trimoxazole (83% vs. 53%), tetracycline (75% vs. 50%), and ampicillin (64% vs. 50%) were comparatively higher among biofilm producers (15).The study performed by Sevanan et al. biofilm-producing organisms are more resistant to antimicrobials. The resistant pattern of antibiotics among biofilm-forming isolates were like amikacin, ampicillin, tobramycin, erythromycin, chloramphenicol, meropenem, and gentamicin was found to be in the order of 71.9, 90.6, 56.3, 59.3, 65.6, 53.1%, 56.3%, and 50.0%. With cephalexin (18.8%) and amoxicillin (37.5%) the resistance was seen least. (15)

In study done by Tajbakhsh E et al, the resistance was maximum to ampicillin (87.5%) among the biofilm producers, followed by other antibiotics like tetracycline (75%), co-trimoxazole (71.25%) and nalidixic acid (72.5%). With nitrofurantoin, 93.75% of biofilm producers were sensitive and 98% of non- biofilm producers were sensitive (24) In the present study, almost all biofilm-producing bacteria demonstrated higher resistance in comparision to the no biofilm counterparts. 37.2% of biofilm producers were resistant to ciprofloxacin while the figure for biofilm non-formers was 28.5%. A similar pattern was found with some of the other antimicrobials used in many studies. It has been noted that hundred times higher antibiotic concentration of antibiotics were required to eradicate biofilm producing organisms than the MIC for the same microorganism in planktonic culture by conventional antimicrobial susceptibility testing (25–27).

The conclusions from the present study shows that various conditions like geographic, clinical and others influences the count of biofilm-producing bacteria. It also understood that higher resistance is shown by the biofilm producers, but biofilm producer are not always resistant to the antibiotics.

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