Comparative Evaluation of Biofilm Detection Methods among Uropathogenic Gram-Positive and Gram Negative Bacteria Isolated from Catheterized Urine Sample and its antibiogram

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Abstract
Background: Biofilm formation is apotential virulence characteristic exhibitedby bacteria resulting in theseverity of many infections. It will give rise to multidrug resistant strains disturbing the effective management of many chronic infections. **Objectives**: The present study aims to focus on the biofilm detection of uropathogenic bacteria by three distinct techniques. The sensitivity and specificity for all the three methods is evaluated. Thepresent study also demonstrates the antibiogram of biofilm producing bacteria. **Materials and Method**: The study includes 89bacterial isolates from catheterized patients with urinary tract infections admitted in ICUs, dialysis units and various wards. Formation of biofilm was detected by: tube method (TM), the tissue culture plate (TCP) method and Congo Red Agar (CRA) method. According to CLSI guidelines the antimicrobial susceptibility test was performed among the biofilm forming bacteria. **Results**: Out of 89 bacterial isolates, 33 were gram positive cocci and 56 were gram negative bacilli. Among the gram positive cocci, 28 (84.8%) andgram negative bacilli, 50(89.2%) formed biofilm. Sensitivity of TCM, TM, and CRM methods were 98.3%, 71.4% and 18.6% respectively. For biofilm forming gram positive bacteria, the maximum antibiotic resistance was achieved towards Penicillin (89%) followed by Amoxyclav (78%). In the case of gram negative bacteria gentamicin showed maximum resistance in 88% followed by Ciprofloxacin in 80% of the isolates. **Conclusion**: Biofilm and multi-drug resistance plays a vital role in thepathogenesis ofcatheterized urinary tract infection (CAUTI). Evaluation of Biofilm among uropathogenic bacteria helps to manage clinically resulting in better prognosis. Tissue culture plate techniqueisvery effectivein detecting biofilm that can be suggested in diagnostic laboratories.
Keywords: Biofilm, virulence factor, multidrug resistance,CAUTI.

Introduction
Urinary tract infections (UTIs) are the frequently encountered infection in the community and hospital settings.[1] In the hospital setting around 40-50% of nosocomial infections are due to UTI.[2] This is highly attributed to extended hospital stay and also imposes economic burden to the patients.[3] In patients receiving indwelling urinary catheters, 15-25% of the patients are susceptible to catheter-associated urinary tract infections (CAUTIs).[4] Generally, urinary catheters are made up of tubular latex or silicone devices and the inserted catheters acquire biofilms on the inner or outer surface. The biofilm formation on the catheter is directly proportional to the time in which the catheter was unchanged.[5]Biofilm is a complex structure with various bacteria adhering to the surface. These adherent bacterial cells become

embedded within polysaccharide matrix. A biofilm is composed of bacterial cells, their extracellular products, and host components.[6]The biofilm in urinary catheters causespersistent infections that complicate antimicrobial therapy. In majority of cases, prolonged catheterization often leads to bacteriuria. Routine treatment in catheterized patients with asymptomatic bacteriuriais not recommended. In symptomatic catheterized patients, catheter changing before urine collection improves the accuracy of urine culture results. This facilitates the patients to respond better to antibiotic therapy as the biofilm which serve as a focus for reinfectionwas removed.[7] The biofilm is a potential virulence factor of bacteria responsible for many prolonged infections. Emergence of multidrug resistant strains among these bacteria often leads to poor clinical approach.[8]

For better prognosis in CAUTIs, it is necessary to detect biofilm among these bacteria. Wide range of uropathogens such as as *E. coli, Proteus, Enterococcus, Pseudomonas*, *Enterobacter, Serratia*, and Candida sppare involved in the indwelling urinary catheters colonization.[9]Uropathogenic*E. coli*are responsible for 80–90% of community-acquired and 30–50% of hospital-acquired urinary tract infections.[10,11]Studies indicate that, biofilm is 1000fold resistant to antibiotics as compared to planktonic cells.[12]Mounting studies have shown that there has been a increasing tendency of antimicrobial resistance among biofilm forming uropathogensto ciprofloxacin, trimethoprim-sulphamethoxazole and Gentamicin.[13] Management of CAUTIs must be carefully followed and effective strategies must be followed based on the antimicrobial susceptibility results and biofilm forming abilityof bacteria.[14]

In this backdrop, the present study demonstratesthebiofilmformationamong the uropathogenicbacteriaby three techniques. A comparative evaluation was done to determine the most accuratetechniqueto detect biofilm among urinary isolates. The study also focuses on the resistance pattern of these bacteria.

Material and Methods

This was descriptive study conducted on 89 bacterial samples isolated from urine samples of catheterized symptomatic patients. The study was conducted during the period between September 2020 to August 2021.

Procedure

Amongthe 89 bacteria,33 were gram positive cocci and 56 were gram negative bacilli and identified using standard techniques. The initial evaluation of isolates obtained from catheterized urine samples of patient was done using standard conventional techniques of microbiological examination. Further, the antimicrobial susceptibility was performed by modified Kirby-Bauer disc diffusion methodusing Muller Hinton agar. Four different tests were used to detect the ability for biofilm formation and they were graded as moderate, high and weak. For every experiment practical aspects, high and moderate biofilm production by all the method was labeled as positive while, that of weak/ no biofilm production was considered negative.

Tissue culture plate technique

In this method, the isolated bacterial were cultured in brain heart infusion (BHI) broth supplemented with 2% sucrose and incubated for 18–24 h at 37°C at a stable position. The broth having visible turbidity was then diluted into 1: 100 and then inoculated using fresh medium. Further, 0.2 ml of the diluted cultures were inoculated into each wells of flat bottom polystyrene plates, and one broth serves as a control for check the sterile condition and nonspecific binding inside the medium. The plates were incubated for 24 hours at 37°C. After incubation with gentle tapping, the content inside the well was removed and were subsequently washed using 0.2 ml phosphate buffer saline (PBS pH 7.2) for 4 times to remove free floating "planktonic" microbes. The sessile adherent bacteria, biofilm producer, were fixed using sodium acetate (2%) for about half an hour and stained with crystal violet (0.1% w/v) for 30 minutes. Excess stain was removed by washing with deionized water and then the plates were allowed to dry. Finally, on every side the cells of bacteria typically shaped the biofilm by means of consist stains with precious stone violet and each well is filled with 95% ethanol to release dye from punctured well. Then, Optical densities (OD) were taken at the wavelength of 570 nm for determining the stained adherent bacteria with the micro-

Enzyme-Linked Immunosorbent Assay (ELISA) auto reader and were graded according to Christensen *et al*[15]. To be precise, the experiment was performed in triplicate.

Tube method

Fromthe overnight culture plates, a loopful of microorganism was inoculated in the tubes containing BHI broth with 2% of sucrose (10 ml) as supplementation. The tubes were incubated for 24 hours at 37°C. Then the culture tubes were allowed for decontamination and washed with PBS (pH 7.3) and allowed to dry. Further, the dried tubes were allowed to stain with crystal violet (0.1%) solution for 30 minutes. The excess stain in the tubes was removed and the dried tubes were observed for biofilm formation. The positive result in biofilm formation was confirmed with the presence of layer including stained material adhered into the inner wall and bottom of the tube, while the stained ring formed as exclusive observed at liquid air interface and it was taken as negative. After the examination of tubes, the amount of biofilm formed was graded as absence, moderate or strong. The tests were performed in triplicate.[15]

Congo Red Agar plate

In this method, the freshly prepared solid medium using BHI broth supplemented with 5% sucrose and Congo red. The microorganism from the inoculated plate was taken which was kept at overnight and was inoculated into the CRA plate. Then the plates were incubated at 37°C for 24 to 48 hours with aerobic conditions. Black colony with a dry crystalline consistency observed and it was considered as a positive result. The experiments were performed in triplicate.[16]

Statistical analysis

McNemer'schisquare test was used for the calculation of sensitivity and specificity.

Results

Among the 33gram positive cocci, TCP detected biofilm in 24 (72.72%)of cocciand it was found to be significant (p=0.03). TM had a detection rate of 18 (54.54%) but it was non –significant (p=0.36). Meanwhile, CRP displayed a detection rate of 11 (33.33%) and found to be non-significant (p=0.81). The results were shown in table 1.

Table 1: Comparison of biofilm detection by TCP, TM and CRA in gram positive cocci

Bacteria	Total	TCP		TM		CRA	
		P	N	P	N	P	N
S. aureus	18	13	5	10	8	6	12
Enterococcus sps	10	7	3	5	5	3	7
CONS	5	4	1	3	2	2	3
p value	33	**0.03***		**0.56^{NS}**		**0.74 ^{NS}**	

(P-Positive, N-Negative, TCP-Tissue culture plate method, TM-Tube adherence method, CRA- Congo red agar method) * denotes statistically significant (p<0.05) and NS-Non-significant.

Among 56 gram negative bacilli, TCPdetected biofilm in 44 (78.57%) of bacilli and it was found to be significant (p=0.02). The detection rate of TM was 32 (55.35%) but found to be non-significant (p=0.36). Meanwhile, the detection rate for CRP was 24 (42.85%) and also found to be non-significant (p=0.81). The results were shown in table 2.

Table 2: Comparison of biofilm detection by TCP, TM and CRA in gram negative bacilli

Bacteria	Total	TCP		TM		CRA	
		P	N	P	N	P	N
E.coli	29	24	5	17	12	14	15
Klebsiella species	19	15	4	11	8	8	10
PsuedomonasAeruginosa	8	5	3	3	5	2	6
p value	56	**0.02***		**0.45^{NS}**		**0.76 ^{NS}**	

(P-Positive, N-Negative, TCP-Tissue culture plate method, TM-Tube adherence method, CRA- Congo red agar method).* denotes statistically significant (p<0.05) and NS-Non-significant

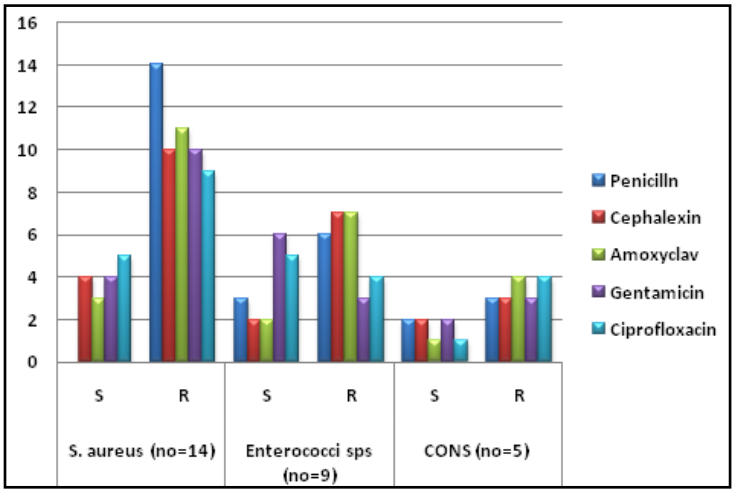
The sensitivity and specificity of three methods was shown in table 3. The TCP displayed highest sensitivity and specificity of 98.3% and 97.2% as compared to TM and CRA. The TP method showed sensitivity and specificity of 71.4% and 88% and CRA method showed sensitivity and specificity18.6% and 83.2% respectively. Among the three methods the accuracy of detecting biofilm formation was least for CRA.

Table 3: Comparisonof sensitivity and specificity of TCP, TM and CRA

Methods	Sensitivity	Specificity
Tissue culture plate	98.3%	97.2%
Tube method	71.4%	88%
Congo red agar	18.6%	83.2%

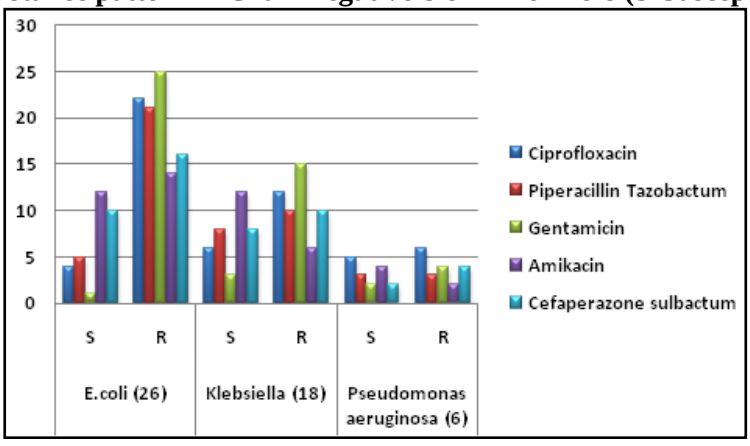
Antibiogram for biofilm producing gram positive cocci was shown in Fig 1.Antibiotics such as Penicillin (10 µg), Cephalexin (30 µg), Amoxyclav (20/10 µg), Gentamicin and Ciprofloxacin (5 µg) was tested. Maximum resistance was observed towards Penicillin (89%) followed by Amoxyclav (78%).

Fig. 1: Antibiotic resistance pattern in Gram positive biofilm formers (S- Susceptible, R- Resistant)



For the evaluation of gram negative bacilli biofilm formers, Ciprofloxacin (5 µg), Piperacillin-Tazobactum (75/30 µg), Gentamicin (10 µg) and Cefoperazone + Sulbactum(75/30 µg) were tested. High resistance was observed for Gentamycin (88%) followed by Ciprofloxacin (80 %). The results were shown in Fig 2.

Fig 2: Antibiotic resistance pattern in Gram negative biofilm formers (S-Susceptible, R- Resistant)



Discussion

The UTI is one of the major causes of bacterial infection in most of the community and hospital settings. In this, CAUTI is the most frequent cause of health-care associated infections and catheter is a predisposing factor to UTI.Studies shows that, on each day of catherization there has been 5% increase in the risk of developing CAUTI and by the end of 30 days, colonization occurs in all the patients.[17]Wide range of studies, substantiates the role of biofilm in the development of CAUTIs.[18]

In this study, biofilm was found in 89.2% of gram negative bacilli which correlate with many other research studies. These organisms play an important role in the CAUTI etiology, since most of them are endogenous microbiota of the perineum with effective biofilm forming capacity. Previous study done by Gunardi et al.[19] displayed that 75% of gram negative bacilli is responsible for biofilm-producing bacteria in the catheter. Similarly in another study done by Niveditha et al.[20] gram negative pathogens around 80% are responsible for the formation of biofilm inCAUTI. In the current study, 84.8 % of gram positive coccideveloped biofilm which is line with the study done by Shrestha et al. where 86% of gram positive cocci are responsible for the biofilm formation in CAUTI.[21]Among gram negative bacilli, biofilm formation was maximum in E.coli (80%) followed by Klebsiella species (77%).Numerous virulence factors are possessed by uropathogenic E.coli for the development of CAUTI such as adhesins, toxins, siderophores, lipopolysaccharide and capsules which facilitate the colonization, invasion and infection of the urinary tract.[22] Our findings is in line with the study done by NagrisSabir et al.[23] where E.coli is the predominant pathogen in the progression of CAUTI. However contrast report is published by Ramadan et al. where the Klebsiella pneumonia is the major biofilm forming organism in CAUTI.[24] In this study, *S.aureus* (78.5%) isthe predominant biofilm forming gram positive bacteriain CAUTI which correlateswith the study done by Murugan et al.[25] where *S. aureus* is the major biofilm formerin patients with indwelling catheters.

In the present study TCP detected biofilm 72.72% of gram positive cocci and 78.57% of gram negative bacilli and it was superior when compared to TP and CRA methods. Similar to the present study report, Halim et al.[26] showed that biofilm production in staphylococci by TCP was higher in blood cultures Blood cultures (82.6%) followed by urine (80%) and body fluids (80%) as compared to TP and CRA methods.

In the present study, the sensitivity and specificity of TCP methods in the detection of biofilm was found to be 98.3 % and 97.2% respectively. This finding is clearly agreeable with Mathur et al.[27] and Oliviera et al.[27](97.1% and 97.6%) in terms of sensitivity.Specificity of TCP is lower than that observed in the previous study (97% and 94.4%). In the current study, the sensitivity and specificity of Tube method was 71.4% and 88% respectively. Similarly, in a study done by Panda et al.[28] Further, in our study the Congo red agar plate method showed sensitivity of 18.6% and specificity of 83.2% and it is in line with Panda et al. with sensitivity and specificity of 16.8% and 93.9% respectively. Based on these findings, tissue culture plate can be taken as a gold standard method for biofilm testing.

Biofilms tolerance antibiotics is might be due to various mechanisms such as low antibiotic penetration, nutrient deprivation adaptive stress responses, decreased metabolism and the development of persister cells. Further, during mixed bacterial growth, the bacteria which are sensitive to antibiotics can be resistant due to the horizontal transfer of plasmid-associated drug-resistant genes from neighboring bacteria within the biofilm.[29]In the present study, majority of biofilm forming gram positive bacteria are resistant to Penicillin (89%) followed by Amoxyclav (78%).Similar studies done by Nargis Sabir et al.[30] showed 100% resistance to Penicillin and in another study done by Awoke et al.[31]66.7% showed resistance to amoxicillinand clavulanic acid. In the case of gram negative uropathogens, maximum antibiotic resistance isobserved for Gentamicin (88%) followed by Ciprofloxacin (80%). Awokeet al.[32] also found that majority of biofilm forming gram negative bacteria were resistantto gentamicin (80.1%) and amoxicillin-clavulanic acid (66.7%). However, higher resistance to cephalosporin and fluoroquinolones was reported from various studies in the range between 56–100% and 66.7–81.1%, respectively.[33]

Presence of biofilm and drug resistance is common in bacteriaassociated withCAUTI. Removal of catheter is vital during the management in these patients but these interventions are often invasive which will affect the quality of life in case of critically ill patients. Currently no licensed agents are available in the management of biofilms. Routine antibiotics are naturally unsuccessful to treat biofilmassociated bacterial infections specifically in critically ill patients. A major recommendation is to develop and implement the minimal biofilm eradication concentration (MBEC) assayfor rapid antimicrobial susceptibility testing for bacterial biofilms in the anticipation that the MBEC assay would be more reliable for selecting suitable antibiotics.

Conclusion

The high prevalence of multidrug resistance among biofilm forminguropathogens is disappointing. For the management of CAUTI, routine surveillance of biofilm formationand antimicrobial resistance is necessary in all cases of symptomatic CAUTI.In the present study, gram negative uropathogens showed maximum biofilm formation (89.2%) when compared to gram positive bacteria. Tissue culture plate techniqueis the most reasonable and reliable strategy for the detection of biofilm with its easy execution methods, cost effectiveness and higher sensitive qualities.More research works are required to find the association of the MDR phenotype in biofilm-producing bacteria, which enable the development of novel therapeutic strategies.

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