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CORRELATION BETWEEN BIOFILM FORMATION AND ANTIBIOTIC RESISTANCE IN UROPATHOGENIC *PSEUDOMONAS AERUGINOSA* CAUSING CATHETER ASSOCIATED URINARY TRACT INFECTIONS

Rouchelle C. Tellis*[1], M. S. Moosabba[2] and Ronald Aloysius Roche[3]

[1]Associate Professor, Department of Microbiology, Yenepoya Medical College, Yenepoya University, Deralakatte, Mangalore-575018, India.

[2]Professor and Head, Department of General Surgery, Yenepoya Medical College, Yenepoya University, Deralakatte, Mangalore-575018, India.

[3]Professor, Department of Microbiology, Malabar Institute of Medical Sciences, Calicut, India.

***Corresponding Author: Rouchelle C. Tellis**

Associate Professor, Department of Microbiology, Yenepoya Medical College, Yenepoya University, Deralakatte, Mangalore-575018, India.

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ABSTRACT

Background and aim: Catheter associated urinary tract infections (CAUTI) accounts for 40% of nosocomial infections in hospitalized patients. *P.aeruginosa* is the third most common pathogen causing hospital acquired CAUTI. As there is paucity of literature about the pathogenesis of UTIs caused by *P.aeruginosa*, this study was undertaken with an aim to assess and correlate biofilm formation with antibiotic resistance in *P.aeruginosa* isolated from catheterized urine samples. **Materials and Methods:** 53 isolates of *P.aeruginosa* obtained from urine of patients with signs and symptoms of CAUTI following semi-quantitative culture on Mac Conkey agar were studied. Bacterial identification was based on colony morphology and standard biochemical tests. Antibiotic sensitivity was tested by Kirby Bauer disc diffusion method. Extended spectrum β-lactamase and metallo-β-lactamase production were detected by phenotypic disc confirmatory test and imipenem-EDTA double disk synergy test respectively. Biofilm formation was studied by microtitre plate assay. **Results:** Of the 53 isolates, 56.6% and 32.1% of were strong and moderate biofilm producers respectively. Among these 72.3% (24/47) were multidrug resistant which was statistically significant compared to weak/non-biofilm producers ($P<0.001$). MDR bacteria belonged to 5 different resistotypes. 21 (39.6%) and 11 (20.8%) isolates were ESBL and MBL producers respectively. **Conclusion:** This is one of the very few studies that has correlated the biofilm formation and antibiotic resistance in uropathogenic P.aeruginosa isolated from cases of CAUTI. Multi drug resistance was found to be significantly higher among the strong biofilm producers than the non-producers which implies that antibiotics should be chosen based on *invitro* antibiotic resistance patterns.

KEYWORDS: Pseudomonas Aeruginosa, Uropathogenic, Biofilm, Multidrug Resistant.

INTRODUCTION

Urinary tract infections (UTIs) are one of the most important bacterial infections affecting individuals across all age groups including young women, children, and the elderly. Studies have shown that approximately 40% of women have had a UTI at some time in their lives.[1] These infections are traditionally classified based on the anatomical site of infection, clinical symptoms, laboratory data and microbiological findings. Based on the site of infection UTIs are categorized as lower urinary tract infections (cystitis and urethritis) upper urinary tract infections (pyelonephritis and urosepsis). Clinically UTIs can be classified as uncomplicated UTIs (acute cystitis and acute pyelonephritis in otherwise healthy individuals without underlying structural abnormalities and co-morbid conditions), complicated UTIs (with structural abnormalities and associated with co-morbid conditions), asymptomatic bacteruria (common in pregnant women) and recurrent UTI.[2] Catheter associated urinary tract infection (CAUTI) is one of the commonest nosocomial infections and bacteria causing these infections are derived from the patient's own colonic flora due to colonization of the indwelling catheter, as these serve as a route of entry for bacteria.[3] The urinary catheters are tubular latex or silicone devices which when inserted and left in situ readily harbour the growth of biofilms on their inner or outer surfaces.[4] Insertion of the catheter itself may damage the mucosal layer, which disrupts the natural barrier of the urinary tract mucosa and initiates bacterial colonization. Organisms can gain entry via extra-luminal route by moving across the outer lumen of catheter or by intra-luminal route by directly entering the interior of catheter.[5] CAUTI accounts for 40% of all nosocomial

infections and are the most common source of gram negative bacteremia in hospitalized patients.[6] The pathogens most frequently associated with CAUTI are *E. coli*, Pseudomonas, Proteus, Enterococcus, Enterobacter, Serratia and Candida spp.[7] *E.coli* being the most common uropathogen associated with CAUTI its pathogenesis and role of virulence factors like adhesins, serum resistance, hemolysins, siderophores etc. in the development of UTIs is well established. *P. aeruginosa* is the third most common pathogen associated with hospital-acquired CAUTI.[3] However there is a paucity of literature about the pathogenesis of UTIs caused by *P.aeruginosa.*

Biofilms play an important role in the pathogenesis of CAUTI, as the catheter surface provides a favorable environment which makes it an ideal site for bacterial a) attachment and biofilm formation.[8,9] One of the most important advantages of biofilms is that of antimicrobial resistance as bacteria forming biofilms are difficult to eradicate due to the antimicrobial resistance phenotype that this structure confers.[10] Bacteria isolated from biofilms can be up to 1000 fold more resistant to antibiotics than planktonic cells due to several mechanisms such as: limitation of antibiotic diffusion through the matrix, transmission of resistance genes by mobile genetic elements, inactivation of the antibiotics by local changes in metal ion concentrations and pH values and presence of dormant but highly tolerant persister cells.[11] *P. aeruginosa* has an innate propensity b) to stick onto the surfaces of catheters and form biofilms **c)** thus causing UTIs in patients with long-term indwelling bladder catheterization. So this study was taken up with the aim to assess and correlate biofilm formation and antibiotic resistance of *P.aeruginosa* isolated from catheterized urine samples.

MATERIALS AND METHODS

Sample collection, bacterial isolation and identification

This was a prospective analytical study conducted over a period of 1 year from April 2013 to March 2014 in the microbiology laboratory of a tertiary care hospital in **d)** South India, to assess the antibiogram and biofilm production in 53 strains of *P.aeruginosa* isolated from indwelling urinary catheters in hospitalized with catheter in situ for >48hrs and clinically showing signs and symptoms of CAUTI. Clearance from the institutional Ethical Committee was obtained and urine samples were collected under aseptic conditions with a sterile syringe from the distal end of the urinary catheter into a sterile urine container and transported to the laboratory immediately. Semi-quantitative culture of urine samples was performed on blood agar and Mac Conkey agar with a calibrated loop to determine colony forming units per ml (CFU/ml). Isolates of *P.aeruginosa* showing a colony count of $>10^3$ CFU/ml or more were included in this study. Bacterial identification was done on the basis of colony morphology and standard biochemical tests.[12]

Antibiotic susceptibility testing

Antibiotic sensitivity of these bacteria was tested by Kirby Bauer disc diffusion method on cation adjusted Muller Hinton agar (MHA) using antibiotic discs from Hi Media Laboratories (India) and the results were interpreted according to the criteria prescribed by Clinical and Laboratory standards institute (CLSI).[13] Antibiotics tested were: amikacin (30µg), cefuroxime (30µg), ceftazidime (30µg), cefipime(30µg), gentamicin (10µg), co-trimoxazole (1.25/23.75µg), ciprofloxacin (5µg), norfloxacin (10µg), netilmycin (30µg), piperacillin (100µg), piperacillin–tazobactum (100/10 µg) and imepenem (10µg). *P. aeruginosa* ATCC 27853 was used as a control strain.

Tests for detection of beta-lactamases

Extended spectrum β-lactamase (ESBL) production was detected using CLSI prescribed phenotypic disc confirmatory test. 13 0.5 Mac Farland's suspension of each isolate was lawn cultured on a MHA plate and ceftazidime(30µg) and ceftazidime / clavulanic acid (30 µg/10µg) discs were placed on the agar plate with a distance of 15mm between the two discs (edge to edge). Cultures were incubated at 37^0C overnight. An observation of ≥ 5mm increase in the diameter of the zone of inhibition for ceftazidime (30µg) when tested in combination with clavulanic acid, versus its zone diameter when tested alone, confirmed the presence of ESBL production.

Metallo-beta-lactamase (MBL) production was detected by Imipenem-EDTA double disk synergy test (DDST) as described by Lee et al.[14] The test organism was lawn cultured on MHA plates and an Imipenem 10µg disk was placed 10mm edge to edge from a blank disc impregnated with 10µl (1900µg) of Ethylenediaminetetraacetic acid (EDTA). After overnight incubation at 37°C an enhancement in the zone of inhibition in the area between Imipenem and EDTA discs in comparison with the zone of inhibition on the far side of the imepenem disc was interpreted as a positive result.

Detection of biofilm formation

The isolates of *P.aeruginosa* were tested for their ability to produce biofilms in microtitre plates according Stepanovic et al.[15] A loopfiul of the overnight bacterial culture was inoculated in 10ml of trypticase soy broth with 1% glucose and incubated at 37°C for 24 h. A 96 well flat bottomed clear polystyrene tissue culture microtitre plate with a lid was inoculated with 200 µl of a bacterial suspension corresponding to 0.5 McFarland with further dilution of 1:100. After 24 h incubation at 37°C, the contents of each well were removed by decantation and each well was washed three times with 300 µl of sterile saline. The remaining attached bacteria were heat-fixed by exposing them to hot air at 60°C for 60 min in the incubator. Then 150 µl crystal violet (2%) stain was added to each well. After 15 min, the excess stain was rinsed off by decantation, and the plate was

washed. 150 µl 95% ethanol was added to each well and after 30 minutes, the optical densities (OD) of stained adherent bacterial films were read using a microtiter plate reader at a wavelength of 620 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). Final OD value of a tested strain was expressed as average OD value of the strain reduced by ODc value (OD=average OD of a strain - ODc). ODc value was calculated for each microtiter plate separately. When a negative value was obtained, it was presented as zero, while any positive value indicated biofilm production. For interpretation of 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

Statistical analysis

The results were expressed by percentages (%) as well as means and standard deviations were calculated for optical density values in biofilm production experiment.

RESULTS

Out of the 53 strains of *P. aeruginosa* isolated from indwelling urinary catheters, 47 were isolated as a single bacterial type with a colony count of $>10^5$ CFU/ml and 6 showed mixed growth of 2 types of bacteria of which *P.aeruginosa* was predominant. 21 (39.6%) isolates were ESBL producers and 11 (20.8%) were MBL producers. Biofilm formation and beta–lactamase production by these isolates is shown in table1.

Table. 1: Biofilm formation and beta – lactamase production in uropathogenic *P.aeruginosa.*

Biofilm formation	No. of isolates	ESBL producers	MBL producers
Strong	30 (56.6%)	18 (60%)	10 (33.3%)
Moderate	17 (32.1%)	3 (17.65)	1 (5.9%)
Weak	4 (7.5%)	Nil	Nil
Negative	2 (3.8%)	Nil	Nil

Resistance to all the antibiotics were comparatively higher among strong biofilm producing *P. aeruginosa* than biofim non-producers (Statistically significant; $P < 0.001$). Majority of the strong biofilm producing *P.aeruginosa* isolates were resistant to fluroquinolones (ciprofloxacin and norfloxacin), co-trimoxazole and third and fourth generation cephalosporins. Higher levels of sensitivity were observed for the following antibiotics: amikacin, piperacillin–tazobactam and cefaperazone–sulbactam. Figure 1 shows the correlation between biofilm production and antibiotic resistance to commonly used anti-pseudomonal antibiotics.

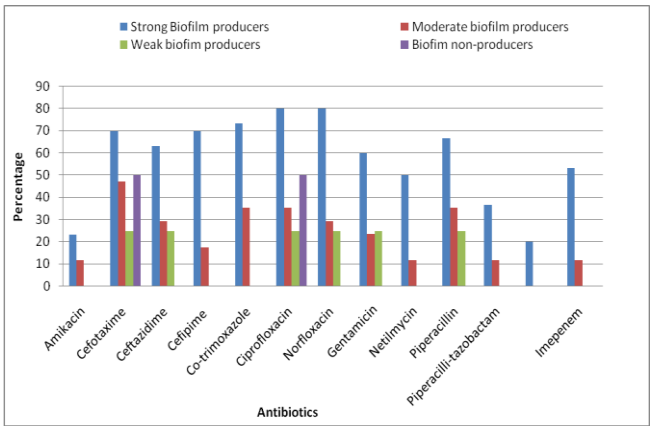

Figure. 1: Antibiotic resistance pattern of biofilm and non-biofilm forming *P.aeruginosa.*

24 (45.3%) isolates of *P.aeruginosa* in this study were multidrug resistant (MDR) and categorised into 5 phenotypes based on their *invitro* resistance to 3 or more different classes of antibiotics as shown in table 2. All the MDR isolates were moderate to strong biofilm producers.

Table. 2: Antibiotic resistance phenotype of MDR *P.aeruginosa* and biofilm formation.

Resistance phenotype	No. of isolates	Resistance profile	Strong biofilm producers	Moderate biofilm producers
Phenotype IA	8	MDR 3^{*} *Caz, Imp, Cipro*	3	5
Phenotype IB	3	MDR 3^{*} *Caz, Pip-taz, Imp*	1	2
Phenotype IIA	3	MDR $4^{¥}$ *Caz, Imp, Ak/Gnt, Cipro*	2	1
Phenotype IIB	6	MDR $4^{¥}$ *Caz, Pip-taz, Ak/Gnt, Cipro*	5	1
Phenotype III	4	MDR $5^{€}$ *Caz, Imp, Pip-taz, Ak/Gnt, Cipro*	3	1

Caz: Ceftazidime, Imp: imepenem, Ak: Amikacin, Pip-taz: Piperacillin- tazobactam, Gnt: Gentamycin, Cipro: ciprofloxacin, MDR: Multidrug resistant

*MDR 3: Resistant to 3 different classes of antibiotics

¥MDR 4: Resistant to 4 different classes of antibiotics

€MDR 5: Resistant to 5 different classes of antibiotics.

DISCUSSION

P. aeruginosa is a successful nosocomial pathogen often associated with device associated hospital acquired infections because of its inherent and acquired resistance to several classes of antibiotics and commonly used disinfectants. Biofilm formation is thought to be a key survival tool especially in relation to catheter and ventilator associated infections. Two salient features of biofilm producing bacteria are increased synthesis of exopolysaccharide (EPS) and the development of antibiotic resistance.[16] As there a very few reports correlating these two factors in uropthogenic *P.aeruginosa* this study was taken up to assess biofilm formation and antibiotic resistance in catheter related UTIs. Clinically a patient with indwelling urinary catheter is said to be suffering from CAUTI if one or more of the following signs and symptoms are seen: fever (temp≥38°C) without any other known cause, urgency or supra-pubic tenderness, pyuria (more than 10 leukocytes/ml of urine) or urine culture showing 10^3 CFU or more/ml of urine, with not more than two types of organisms.[17] CAUTI being the most common nosocomial infection needs to be reported to the hospital infection control team and the rate of CAUTI is an important indicator of the quality of patient care.

In this study, 56.6% and 32.1% of the isolates were strong and moderate biofilm producers respectively, showing their propensity to form biofilms and colonise the urinary catheters. High rates of antibiotic resistance were noted among these biofilm producers. Similarly high rates of biofilm formation and antibiotic resistance were observed by Carlos J et al. who reported biofilm formation in 83% of clinical strains of *P.aeruginosa* and antibiotic resistance of 75% to ciprofloxacin, 67% to ceftazidime,100% to ceftriaxone among the biofilm producers.[18] A study by Gurung J et al on the association of biofilm production with multidrug resistance among clinical isolates of *P.aeruginosa* isolated from patients in intensive care unit showed that 33% of these isolates were biofilm producers and also showed high rates of antibiotic resistance (ranging from 63% to 81%) to commonly used antibiotics like ceftazidime, cefoperazone, ofloxacin, amikacin, ciprofloxacin, and ceftriaxone.[19] This study shows that amikacin, cefaperzone-sulbactam and piperacillin tazobactam could be the antibiotics of choice for treating CAUTI caused by *P.aeruginosa.* Higher rate of resistance to imepenem (33.9%) was observed in our study and 20.85% of the isolates were MBL producers. A similar finding has been reported by Chand AE from North India with imepenem resistance of (36.1%) and 34.1% being MBL producers.[20] Higher rates of imepenem resistance could be attributed to the local antibiotic prescribing practices. In our study *P. aeruginosa* showed higher sensitivity to amikacin (83.1%) which is also similar is the finding of Javiya AV *et al.*[21]

The present study also showed significant correlation between biofilm production and multidrug resistance, where in 72.3% (24/47) of strong and moderate biofilm producers were multidrug resistant belonging to 5 different phenotypes. Gurung J *et.al.* have reported that 57% of *P. aeruginosa* which were biofilm producers were multi drug resistant.[19] Therefore antibiotic therapy for CAUTI should always be guided by *invitro* antibiotic sensitivity testing, as increasing numbers of nosocomial pathogens are showing multidrug resistance. Monotherapy with antibiotics such as beta-lactams especially anti-pseudomonal 3rd and 4th generation cephalosporins which are only active against dividing *P. aeruginosa* cells, are not very efficient in eradicating biofilms. Neil *et al* reported that colistin is the only antimicrobial active against the non-dividing central part of *P. aeruginosa* biofilms *invitro.* Since the metabolically active surface layer of the biofilm is susceptible to ciprofloxacin in contrast to the dormant central part of the biofilm, combination therapy with ciprofloxacin and colistin was able to kill all cells in the biofilm.[10]

CONCLUSION

A greater understanding of the pathophysiology of uropathogenic *P.aeruginosa* in biofilm formation and their role in serious catheter related infections will help in development of new and more effective treatment modalities resulting in improved patient management. As the present study shows a strong correlation between biofilm production and MDR it is strongly recommendable that antibiotic therapy for CAUTI should be guided by *invitro* antibiotic sensitivity testing. A fair knowledge of the local antibiotic resistance trends among these hospital acquired pathogens could help us formulate a robust antibiotic policy. Biofilms can be prevented by early aggressive antibiotic prophylaxis to eradicate planktonic growth or they can be treated by chronic suppressive therapy. A promising strategy could be the use of enzymes that can dissolve the biofilm matrix (e.g. DNase and alginate lyase) as well as quorum-sensing inhibitors that will increase biofilm susceptibility to antibiotics.

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