Research Article

Study of biofilm production and antimicrobial sensitivity pattern of uropathogens in a tertiary care hospital in North India

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ABSTRACT

Background: Urinary tract infection (UTI) is one of the most common infectious diseases encountered in clinical practice. Emerging resistance of the uropathogens to the antimicrobial agents due to biofilm formation is a matter of concern while treating symptomatic UTI. But studies addressing the issue of biofilm production by uropathogens in Indian scenario are scarce. Aim of the study was to study biofilm formation and antimicrobial sensitivity pattern of uropathogens.

Methods: Prospective observational study conducted in a tertiary care hospital. Total 300 isolates from urinary samples were analysed for biofilm formation by tissue culture plate method. We compared the antimicrobial sensitivity pattern of the biofilm producing and non-producing uropathogens. For statistical analysis Chi-square test was applied when two or more set of variables were compared. A 'p' value of <0.05 as considered to be statistically significant.

Results: Biofilm formation was detected in 137 (45.6%) isolates. All the biofilm producing organisms were found to be multi drug resistant (MDR). Biofilm producing gram negative organisms were sensitive to imipenem, piperacillin-tazobactam and netilmicin. While, vancomycin, linezolid, rifampicin and nitrofurantoin are agents effective against biofilm producing gram positive organisms.

Conclusions: Biofilm production by uropathogens is a common phenomenon now a days and it is one of the important mechanisms of antimicrobial resistance among the uropathogens.

Keywords: Biofilm, Uropathogen, Antimicrobial sensitivity, North India

INTRODUCTION

Currently, urinary tract infection (UTI) is one of the most common infection encountered in clinical practice and antimicrobial resistance is a serious concern in treatment of symptomatic UTI.¹ Bacteria attach to surface aggregate in a hydrated polymeric matrix of their own synthesis to form biofilms.² High antimicrobial concentrations are required to inactivate organisms growing in a biofilm, as antibiotic resistance can increase by 1,000 folds.³ So, the primary objective of this study was to detect biofilm formation by uropathogens. We also studied the

antimicrobial sensitivity pattern of the biofilm producing organisms.

METHODS

The current study was prospectively conducted in a tertiary care institution over a period of 1 year. Out of 14827 urine samples received in the laboratory during this period, 2321 samples (15.6%) showed bacterial growth (detected by standard conventional microbiological techniques). A total of 300 isolates obtained from those samples were analysed further for

biofilm production by tissue culture plate (TCP) method (described below).⁴

In TCP method, isolates from fresh agar plates were inoculated in brain heart infusion (BHI) broth with 2% sucrose and incubated for 18-24 hours at 37°C in stationary condition. The broth with visible turbidity was diluted to 1 in 100 with fresh medium. Individual wells of flat bottom polystyrene plates were filled with 0.2 ml of the diluted cultures and only broth served as control to check sterility and non-specific binding of medium.

These plates were incubated for 24 hours at 37°C. After incubation, the content of the well was gently removed and then were washed four times with 0.2 ml of phosphate buffer saline (PBS pH 7.2) to remove free-floating 'planktonic' bacteria.

Biofilms formed by adherent 'sessile' organisms in plate were fixed with sodium acetate (2%) for half an hour and stained with crystal violet (0.1% w/v) for half an hour. Excess stain was rinsed off by thorough washing with deionised water and plates were kept for drying. Adherent bacterial cells usually formed biofilm on all side wells and were uniformly stained with crystal violet.

Optical densities (OD) of stained adherent bacteria were determined with a micro ELISA auto reader at wavelength of 570 nm (OD 570 nm) and were graded as per Christensen et al (Table1).⁴ These OD values were considered as an index of bacteria adhering to surface and forming biofilms.

The experiment was performed in triplicate and repeated three times. For all practical purpose strong and moderate biofilm production was considered as positive and weak or no biofilm formation was considered negative. American Type Culture Collection (ATCC) strain *S. aureus* (ATCC 25923), *E. coli* (ATCC 25922) and *P. aeruginosa* (ATCC 27853) were used as positive control strains.

All isolates were subjected to antibiotic susceptibility testing using Kirby-Bauer disc diffusion method, done on Mueller-Hinton Agar (MHA) plate as per the Clinical and Laboratory Standard Institute (CLSI) guidelines.

American Type Culture Collection (ATCC) strain *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853) were used as control strains.^{5,6} The antibiotic sensitivity was compared between biofilm forming and non-biofilm forming organisms.

The statistical analysis was carried out using SPSS software version 16.0. Data were presented as percentages and proportions. Chi-square test was applied when two or more set of variables were compared. The critical value of 'p' indicating the probability of significant difference was taken as <0.05.

RESULTS

Out of the 300 isolates (100 *Esch. coli*, 50 *Klebsiella* spp., 50 *Pseudomonas* spp., 30 *Staphylococcus* spp., 20 *Proteus* spp., 20 *Enterobacter* spp., 15 *Citrobacter* spp., 10 *Acinetobacter* spp., and 5 *Enterococcus* spp.), 265 (88.3%) were gram negative and 35 (11.7%) were gram positive organisms.

Table 1: Classification of bacterial adherence by tissue culture plate method.

Mean optical density (OD) values	Adherence	Biofilm formation
<0.120	None	None / weak
0.120-0.240	Moderate	Moderate
≥ 0.240	Strong	High

Biofilm formation was detected in 137/300 (45.6%) isolates. *E. coli* (36 out of 137; 26.3%) and *S. aureus* (13 out of 137; 9.5%) were the most common biofilm producing organisms in our study. All the biofilm producing organisms were found to be multi drug resistant (MDR).

Table 2: Antibiotic resistance pattern of biofilm forming (BF) and non-biofilm forming (NBF) gram negative bacteria other than non-fermenters (n=205).

Antibiotics	Resistance (%)		P value
	BF (n=82)	NBF (n=123)	
Ampicillin	100	98.3	0.26
Amoxycillin-clavulanic acid	100	94.3	0.28
Piperacillin-tazobactam	62.1	17	<0.00001
Gentamicin	40.2	38.2	0.77
Amikacin	87.8	33.3	<0.00001
Netilmicin	81.7	34.9	<0.00001
Ceftazidime	87.8	51.2	<0.00001
Cefepime	81.7	39.8	<0.00001
Ceftriaxone	87.8	36.5	<0.00001
Cefuroxime	89.02	46.3	<0.00001
Cefazolin	92.6	49.5	<0.00001
Ciprofloxacin	82.9	56	<0.00001
Norfloxacin	71.9	72.3	0.95
Ofloxacin	81.7	54.4	<0.00001
Imipenem	26.8	19.5	0.22
Meropenem	81.7	17.8	<0.00001
Aztreonam	100	91	0.005
Cotrimoxazole	81.7	65.8	0.013
Doxycycline	95.12	47.9	<0.00001
Nitrofurantoin	47.5	27.6	0.004

*BF=Biofilm forming, †NBF=Non -biofilm forming.

For depicting the antimicrobial resistance, we categorised the organisms as gram positive organisms, non-

fermenters (*P. aeruginosa* and *A. baumannii*) and gram negative organisms other than non-fermenters. Of the total 205 isolates of gram negative organisms other than non-fermenters, 82 (40%) were BF. The antibiotic susceptibility pattern of BF and NBF gram negative organisms other than non-fermenters is shown in Table 2. All (100%) the BF isolates were resistant to ampicillin, amoxycillin-clavulanic acid and aztreonam. There was

insignificant difference in sensitivity to ampicillin, amoxycillin-clavulanic acid, gentamicin, norfloxacin and imipenem between the BF and NBF. The BF isolates were mostly sensitive to imipenem, gentamicin and nitrofurantoin. Imipenem was the only antibiotic having least resistance among the BF and NBF isolates.

Table 3: Antibiotics resistance pattern of biofilm forming (BF) and non-biofilm forming (NBF) *Pseudomonas Aeruginosa* (n=50).

Antibiotics	Resistance (%)		P value
	BF (n=29)	NBF (n=21)	
Ceftazidime	93.1	85.7	0.390
Cefepime	75.8	57.1	0.161
Gentamicin	72.4	28.5	0.002
Amikacin	55.1	9.5	0.001
Netilmicin	65.5	42.8	0.111
Imipenem	27.5	0	0.009
Meropenem	48.2	14.2	0.012
Piperacillin-Tazobactam	55.1	14.2	0.003
Aztreonam	100	85.7	0.036
Ofloxacin	75.8	66.6	0.475
Norfloxacin	79.3	38	0.003
Ciprofloxacin	68.9	57.1	0.390

*BF=Biofilm forming, †NBF=Non –biofilm forming.

Table 4: Antibiotics resistance pattern of biofilm forming (BF) and non-biofilm forming (NBF) *Acinetobacter Baumannii* (n=10).

Antibiotics	Resistance (%)		P value
	BF (n=7)	NBF (n=3)	
Ciprofloxacin	71.5	33.3	0.260
Ceftazidime	85.7	33.3	0.098
Cefepime	85.7	33.3	0.098
Ceftriaxone	71.5	33.3	0.260
Gentamicin	71.5	66.6	0.880
Amikacin	57.1	66.6	0.778
Netilmicin	14.2	66.6	0.098
Imipenem	28.5	66.6	0.260
Meropenem	57.1	66.6	0.778
Piperacillin- Tazobactam	14.2	66.6	0.098
Doxycycline	71.5	0	0.038
Cotrimoxazole	85.7	0	0.011

*BF=Biofilm forming, †NBF=Non –biofilm forming.

Among the 50 *P. aeruginosa* (PA) and 10 *A. baumannii* (AB) isolates 58% of PA and 70% of AB isolates were BF. The antibiotic sensitivity pattern of BF and NBF *P. aeruginosa* (PA) and *A. baumannii* (AB) is shown in Table 3 and 4 respectively.

All the biofilm producing PA and AB isolates were multi drug resistant (MDR). Both PA and AB isolates were highly resistant to ceftazidime, cefepime, gentamicin and

ciprofloxacin. The PA isolates had highest sensitivity to imipenem, but AB showed maximum sensitivity to piperacillin- tazobactam and netilmicin followed by imipenem. Of the total 35 gram positive isolates (26 *S. aureus*; 4 Coagulase negative staphylococcus (CONS); 4 *E. faecalis* and 1 *E. faecium*), 19 isolates (13 *S. aureus*, 3 CONS and 3 *E. faecalis*) were BF. All the BF and NBF gram positive isolates were sensitive to vancomycin, linezolid and rifampicin (Table 5).

The BF isolates were highly resistant to penicillin, gentamicin, erythromycin and ciprofloxacin. Of the other

antimicrobials, the BF isolates had least resistance to nitrofurantoin.

Table 5: Antibiotics resistance pattern of biofilm forming (BF) and non-biofilm forming (NBF) gram-positive bacteria (*staphylococcus spp. and enterococcus spp.*) (n=35).

Antibiotics	Resistance (%)		P value
	BF (n=19)	NBF (n=16)	
Penicillin	94.7	75	0.096
Ciprofloxacin	78.9	75	0.782
Norfloxacin	52.6	56.2	0.830
Doxycycline	52.6	62.5	0.557
Erythromycin	84.4	62.5	0.058
Gentamicin	94.3	68.7	0.127
Nitrofurantoin	31.5	25	0.929
Linezolid	0	0	NA*
Rifampicin	0	0	NA*
Vancomycin	0	0	NA*

*NA: Not Applicable, †BF=Biofilm forming, ‡NBF=Non –biofilm forming.

DISCUSSION

Biofilm producing bacteria are responsible for many refractory infections including UTIs and are particularly difficult to eradicate. Antimicrobial resistance is a leading concern and efforts should be made to ensure an appropriate therapy for symptomatic UTI.¹

In the current study, amongst the gram negative bacteria other than non-fermenters, the BF isolates showed high resistance to ampicillin, amoxycillin-clavulanic acid and aztreonam as compared to NBF isolates. Imipenem was the most effective drugs against both BF and NBF. Sharma et al have shown that the susceptibility pattern of the BF isolates of *E. coli* ranged from 16-57% while that of the NBF isolates ranged from 38-76%.⁷

They found that amoxycillin-clavulanic acid and nitrofurantoin were the most effective drugs against the BF isolates while the NBF isolates were mostly sensitive to nitrofurantoin, amoxycillin-clavulanic acid and ceftizoxime.⁷ Hassan et al also found that the BF isolates were more resistant to the antibiotics as compared to the NBF bacteria.⁸ They found that the isolates were 100% resistant to ampicillin.

However the resistance to ciprofloxacin (95%) was more in the BF isolates as compared to the present study (82.9%). Their isolates were not resistance to meropenem, while in our study meropenem resistance was 81.7% and 17.8% in BF and NBF isolates respectively.

The result of other drug used by the author were comparable [amikacin (64% vs 37%) and ceftriaxone (58% vs 33%), Aztreonam (90% vs 50%) for the BF and NBF isolates respectively].⁸ For the *P. aeruginosa*

isolates, resistance to all the drugs was more in case of the BF isolates and imipenem was the most effective drugs amongst the BF isolates. All (100%) the PA isolates in the study by Nagaveni et al were resistant to ceftazidime, ceftazidime and ciprofloxacin, which is higher than that in our study.⁹

However, the resistance of biofilm producing PA to imipenem has been reported to be 20% which is similar to our study result (27.5%). In another study by Gurung et al the resistance to aminoglycoside, fluoroquinolones, and β -lactam group of antibiotics was significantly more in BF *P. aeruginosa* as compared to NBF (>55% vs. >20%; $p<0.001$).^{9,10}

In case of *A. baumannii*, both the BF isolates showed similar resistance to ceftriaxone, doxycycline and gentamicin (71.5%). The resistance to ciprofloxacin was high in BF (71%) than the NBF (33.3%) isolates ($p=0.260$). The most effective drugs for the BF AB were piperacillin-tazobactam, netilmicin and imipenem (28.5%).

According to Rao et al norfloxacin (11.7%) ceftazidime (32.3%) and ceftriaxone (35.2%) were more effective in the BF isolates while in present study ceftazidime (33.3%) and ceftriaxone (33.3%) were effective mostly in the NBF isolates.¹¹

The resistance to ceftazidime, ceftazidime by all the AB isolates in the study by Rao et al was 30.9%, 36.3% respectively which is lower than that in present study.¹¹ However, ciprofloxacin resistance in BF in their study was similar to our study (72.7%).¹¹

In a study by Bano et al the most effective drug against the BF AB was imipenem (25%). They could not find

significant difference in resistance pattern of doxycycline between BF and NBF (65% vs 60%) ceftazidime (73% vs. 83%), and gentamicin (80% vs. 77%).¹² While in present study it was (71.5% vs 0%) for doxycycline, (85.7% vs 33.3%) for ceftazidime and (71.5% vs 66.6%) for gentamicin. However, ciprofloxacin resistance (66%) in their study was similar to that of our study. The resistance of biofilm producing AB to netilmicin has been reported to be 27.5% which different from present study result (14.2%).¹¹

Amongst the gram positive bacteria the BF isolates were more resistant to penicillin (94.7%) as compared to NBF isolates (75%) while the resistance to ciprofloxacin was similar in both the groups. Linezolid, vancomycin, and rifampicin were 100% effective against both the groups. All the organisms (100%) in the study by Hassan et al were resistance to penicillin.⁸ Similar to our finding they also found that linezolid and vancomycin to be most effective drugs against BF isolates.

We found ciprofloxacin resistance to be >70% in present study which is in contrast to previous reports by Nwanz et al (37.5%) and similar (70%) to that reported by study by Manjunath et al.^{13,14}

The resistance of gentamicin as shown by Manjunath et al was low (17.6%) while in the current study it was 80%.¹⁴ Nwanz et al reported nitrofurantoin resistance to be 42% which was higher than that in our study (31.5%).¹³ So, nitrofurantoin was found to be an effective drug in treatment of gram positive UTIs.

CONCLUSION

To conclude, biofilm production by uropathogens is a common phenomenon now days. In our study, antibiotic resistance was significantly higher in biofilm producing organism re-emphasising the role of biofilm production in spreading multiple drug resistance (MDR) amongst the uropathogens.

However, MDR in non-biofilm producing organisms emphasizes upon the fact that though biofilm production is common among the uropathogens, it is not the only reason behind emergence of MDR organisms and it is essential to look for other mechanisms that are conferring multidrug resistance in these isolates.

Imipenem, piperacillin- tazobactam and netilmicin are the few antimicrobial agents that are effective against both BF and NBF gram negative organisms while, vancomycin, linezolid, rifampicin and nitrofurantoin are agents effective against BF and NBF gram positive organisms.

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Ethical approval: The study was approved by the Institutional Ethics Committee

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