

Study of biofilm formation and its correlation with drug resistance among *E.coli* positive samples in catheter-associated urinary tract infection

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ABSTRACT

The present study was aimed to perform *in vitro* detection of biofilm formation among *E. coli* positive samples and to correlate the biofilm production with antibiotic resistance pattern. Samples were collected from patients of all age groups and both sexes with a urinary catheter for at least two days suffering from symptoms of UTIs and were characterized by routine bacteriological methods. Ninety six wellmicrotiter-plate tests were done to determine *in vitro* biofilm formation. Antibiotic susceptibility was determined by using standard Kirby–Bauer disk diffusion method in accordance with Clinical and Laboratory Standards Institute guidelines.

In our study, among 120 *E. coli* isolates for UTIs in catheterized patients; One hundred and four isolates were biofilm producers, among which 64 (53.33%) were weak biofilm producers, followed by 33 (27.50%) moderate and 7 (5.83%) strong. 16 (13.33%) isolates were non biofilm producers. The correlation between biofilm producer and non-biofilm producer with antibiotic resistance was found statistically significant with ($P < 0.05$) for ampicillin, ceftriaxone, cefotaxime, ciprofloxacin, cotrimoxazole, norfloxacin, gentamicin, piperacilin tozobactam, cefoperazone + sulbactam, amikacin and meropenem. This study enhances understanding of biofilm detection and antibiotic resistance in *E. coli*. Conclusively it is helpful in the development of newer and more effective treatment in Catheter associated uterinetract infection (CAUTI) patients.

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KEY WORDS : Antibiotic resistance, Biofilm, CAUTI, *Escherichia coli*, ICU, Urinary tract infection

Introduction

The rising occurrence of UTI in ICUs is exaggerated by the high frequency of urinary catheterization, recurrent contact with health care workers and augmented resistant pathogens¹⁰. CAUTI is found to happen in 50% of cases once the patient is under Catheterization for over 5 days.¹⁷ In Indian population, every age group is affected by catheter-associated urinary tract infection (CAUTI) and it is a noteworthy source of morbidity and mortality.⁷

Bacteria cling to the surfaces, firstly in a reversible association and then through irreversible attachment, and eventually develop into an adherent biofilm of highly structured and cooperative consortia.^{3,9,21} Features are an augmented resistance to antibiotic treatment, perseverance, dodging of host immune systems (thus displaying a transformed immune response), expression of changed proteins and of quorum-sensing molecules.

Biofilm is a microbiologically consequent sessile community categorized by cells irrevocably attached to

a substratum or to each other and entrenched in a medium of extracellular polymeric substances produced by them.⁴ The biofilm formation is arbitrated by mechanical, biochemical and genetic factors¹. It encourages encrustation and shields bacteria from the hydrodynamic forces of urine flow, host defenses and antibiotics.¹⁹ Biofilms increase pathogen virulence and play a potential role in innumerable infections; they are currently estimated to be accountable for over 65% of nosocomial infections (NI) and 80% of all microbial infections.¹² The occurrence of biofilm among uropathogenic *E. coli* (UPEC) ranges from 60% to 70%.¹⁴⁻¹⁶

Biofilms have major medical significance as the susceptibility to the antimicrobial agents get decreased by them. Additionally, the closeness of cells within a biofilm can enable a plasmid exchange thereby enhancing antimicrobial resistance.²⁰

UTI severity is dependent on the bacterium and host sensitivity virulence.¹³ Biofilm formation in *E. coli*

requires a set of gene expressions enabling its instigation, attachment and subsequent maturation.

In modern clinical microbiology, bacterial biofilms formation is considered as pathogenicity trait. Worldwide increase has been recorded in antibiotic resistance of urinary tract pathogens.¹⁵ Microorganisms growing in a biofilm are inherently more resistant to antimicrobial agents than planktonic cells.

Antibiotic resistance of organisms growing in a biofilm can increase by 1,000 folds, hence in order to inactivate them, high antimicrobial concentrations are required.¹⁸

In this context, the present study is intended to undergo *in vitro* detection of biofilm formation among *E. coli* strains isolated from urine cultures of catheterized patients by microtitre plate method and to correlate the biofilm production with antibiotic resistance pattern.

Materials and Methods

This hospital based prospective cross-sectional study was conducted over a period of 1 year (November 2017 to November 2018) in Clinical Microbiology Laboratory of Rama Medical College, Kanpur. A total of 120 *E. coli* strains were isolated from urine samples of catheterized patients.

The following patients were found suitable for the study :

Inclusion Criteria: Urine samples were collected from patients of all the groups and both the sexes, with indwelling urinary catheters for at least 2 days, who were suffering from the symptoms of UTIs (fever > 38°C, urgency, frequency dysuria or suprapubic tenderness).

Exclusion Criteria: Non-catheterized patients with urinary tract infection, patients undergoing treatment for UTI when the catheter was inserted; and patients with symptoms of UTI prior to the catheterization were excluded.

The samples were collected with sterile syringes from the distal ends of the urinary catheters under complete aseptic conditions and were transferred to sterile urine containers. The urine samples were inoculated onto the Cystine Lactose Electrolyte Deficient (CLED) medium with calibrated loop to determine the Colony Forming Units (CFU). Colony morphology, gram staining and the standard biochemical tests were done for identification of the isolates. Antibiotic susceptibility was determined by using standard Kirby–Bauer disk diffusion method in accordance with Clinical and Laboratory Standards Institute guidelines.²

The Detection of the Biofilm Formation: For each isolate, a few colonies were suspended in a test tube containing 5 ml of Luria Bertany (LB) and this was

incubated at 37°C for 18 to 24h. After this period, 1 ml of bacterial suspension was inoculated into a different test tube containing 10 ml of sterile LB medium. Three wells of a sterile 96-well flat-bottomed plastic tissue culture plate with a lid were filled with 200 µl of each bacterial suspension. Negative controls (blank) were LB broth alone, which were dispensed into eight wells per tray. After stationary aerobic incubation at 37°C for 24h, the content of the wells were carefully drawn off and each well was washed three times with 250 µl of sterile physiological saline. The plates were shaken to remove all non-adherent bacteria.⁸

The remaining attached bacteria (biofilm) in the wells were fixed with 200 µl of methanol 99% for 15 min, which was then discarded. The wells were flicked and let air dry in an inverted position (room temperature, about 30 min). Biofilms were stained with 0.2 ml of crystal violet 2% (used for Gram staining) for 5 min at room temperature. Excess stain remaining in the wells were rinsed out by placing the plate under running tap water. Afterwards, the plates were inverted on a towel and let air dry. The dye bound to the adherent cells in the each well was resolubilized with 160 µl of glacial acetic acid 33% to quantify biofilm production. The lidded plates were left at room temperature for 30 min. Afterward, the optical density (OD) of resolubilized crystal violet in each well was measured at 570 nm (OD₅₇₀) using an Elisa reader. The reading was performed twice: (i) before addition of glacial acetic acid, as a standard microtiter-plate test and (ii) after addition of glacial acetic acid. We have introduced a classification of adherence capabilities of tested isolates into four categories for a comparative analysis of test results. All isolates were classified into the following categories: non-adherent (-), weakly (+), moderately (++) and strongly (+++) adherent, based on the ODs of bacterial biofilms. The cutoff OD value (OD_c) was defined as three standard deviations above the mean OD of the negative control. Isolates were classified as follows:

$OD \leq OD_c$ = not a biofilm producer (-), $OD_c < OD \leq 2 \times OD_c$ = weak biofilm producer (+), $2 \times OD_c < OD \leq 4 \times OD_c$ = moderate biofilm producer (++) , $4 \times OD_c < OD$ = strong biofilm producer (+++) [8]. All tests were carried out 3 times and the results were averaged.

Statistical analysis was done for Chi square(+2), P value, and correlation coefficient (r) using InStat Software. P < 0.05 was considered as statistical significant.

Result

Biofilm formation:

The results have shown that from 120 *E. coli* isolates for UTIs in catheterized patients; 104 isolates

TABLE-1 : Biofilm formation

| Biofilm | Grade | No. of Isolates | Percentage |
|--------------------|--------------|-----------------|----------------|
| Producing Isolates | Strong | 7 | 5.83% |
| | Moderate | 33 | 27.50% |
| | Weak | 64 | 53.33% |
| | Total | 104 | 86.67% |
| Non-Producers | | 16 | 13.33% |
| | Total | 120 | 100.00% |

were biofilm producers, among which 64 (53.33%) were weak biofilm producers, followed by 33 (27.50%) moderate and 7 (5.83%) strong. 16 (13.33%) isolates were non biofilm producers by TCP method(Fig. 1).

Antibiotic resistance pattern of *E.coli* (Biofilm producers Vs Non-producers):

It was found that the biofilm producing isolates displayed relatively high resistance against tested antibiotics as compared to non-producers. (As shown in the Table-2). Among the biofilm producers, maximum resistance was seen to Ampicillin(85%),ceftriaxone 80%, cefotaxime 79.17%, ciprofloxacin 79.17%. Minimum resistance was seen to imipenem (10%), nitrofurantoin (10%) followed by, Meropenem(30%). But among the nonbiofilm producers, all antibiotics were sensitive. The correlation between biofilm producer and nonbiofilm producer with antibiotic resistance was found statistically significant with ($P<0.05$) for ampicillin, ceftriaxone, cefotaxime, ciprofloxacin, co trimoxazole, norfloxacin, gentamicin, piperacilin tozobactam, cefoperazone + sulbactam, amikacin & meropenem. The correlation could not be found to be significant for rest of the antibiotics .

Discussion

In present study the results have shown that from 120 *E. coli* isolates for UTIs in catheterized patients; 104 (86.66%) isolates were biofilm producers. This is similar to an study in which among 172 cystitis isolates, 145 (84.3%) produced biofilm¹¹. Our results are in contrast with an earlier study¹⁶ where they have documented the biofilm productive *E. coli* isolates to be as less as (67.5%) whereas others reported a higher rate of biofilm production of 92%.

Among 104 biofilm producer isolates in our study, 64 (61.54%) were weak biofilm producers, followed by 33

(31.73%) moderate and 7 (6.73%) strong. These results are similar to the previous study⁶ where among 53 biofilm producer isolates: 36 were weakly biofilm productive isolates with a percentage of (67.92%) followed by (15) isolates which were moderate in their production (28.30%), only (2) isolates were strongly formed biofilm (3.77%). Most were weak biofilm producers (88, 60.7%) and only few were moderate (44, 30.3%) or strong (13, 9.0%) producers.

In a previous study the resistance of UPEC biofilm producers isolates towards:(ciprofloxacin, ceftriaxone, piperacillin, nalidixic acid, amoxicillin-clavulanic acid, gentamycin, azithromycin, imipenem, amikacin) were (94.44, 92.6, 90.2, 86.3, 86.3, 97.05, 85.3, 100 and 75)% respectively, whereas the resistance of non- biofilm producers were 5.55, 7.4, 9.8, 13.7, 13.7, 2.95, 14.7, 0,25 and 0.0 % respectively.⁶ Similar to this, in our study the biofilm producing isolates displayed relatively high resistance against tested antibiotics as compared to non-producers: Ampicilline 85%, piperacilline tozobactm 45.83%, ceftriaxone 80%, cefotaxime79.17%, ciprofloxacin 79.17%,norfloxacin 62.5%,amikacin 32.50%, gentamycin 51.67%,cotrimaxazole 64.17%, cefoperazone 44.17%, imipenem10%, meropenem 30%, nitrofurantoin 10% respectively. In biofilm non producers it is 4.17%,0%,3.33%,4.17%,0.83%, 0.83%, 0.83%, 0.83%,5%,0%,0.83%, 0.83%, 0.83%.

In a study the correlation between biofilm producer and nonbiofilm producer with antibiotic resistance was found statistically significant with ($P = 0.01$) for antibiotics: co trimoxazole, norfloxacin, gentamicin, ciprofloxacin, nalidixic acid, cephalixin, imipenem, and amoxiclav¹¹; where as in our study it is found significant with ($P<0.05$) for ampicillin, ceftriaxone, cefotaxime, ciprofloxacin, cotrimoxazole, norfloxacin, gentamicin, piperacilin

TABLE-2 : Antibiotic resistance pattern of *E.coli* among biofilm producers and biofilm non-producers

| Antibiotic | Biofilm | | | | Total | Resistant Percentage | P Value |
|------------|------------------------------------|------------|------------------------------|------------|-------|----------------------|---------|
| | Biofilm producing isolates (n=104) | Percentage | Biofilm Non-producers (n=16) | Percentage | | | |
| AMP | 102 | 85.00% | 5 | 4.17% | 107 | 89.17% | 0.000 |
| PIT | 55 | 45.83% | 0 | 0.00% | 55 | 45.83% | 0.000 |
| CTR | 96 | 80.00% | 4 | 3.33% | 100 | 83.33% | 0.000 |
| CTX | 95 | 79.17% | 5 | 4.17% | 100 | 83.33% | 0.000 |
| CIP | 95 | 79.17% | 1 | 0.83% | 96 | 80.00% | 0.000 |
| NOR | 75 | 62.50% | 1 | 0.83% | 76 | 63.33% | 0.000 |
| AMK | 39 | 32.50% | 1 | 0.83% | 40 | 33.33% | 0.014 |
| GEN | 62 | 51.67% | 1 | 0.83% | 63 | 52.50% | 0.000 |
| COT | 77 | 64.17% | 6 | 5.00% | 83 | 69.17% | 0.003 |
| CFS | 53 | 44.17% | 0 | 0.00% | 53 | 44.17% | 0.000 |
| IPM | 12 | 10.00% | 1 | 0.83% | 13 | 10.83% | 0.526 |
| MERO | 36 | 30.00% | 1 | 0.83% | 37 | 30.83% | 0.022 |
| NIT | 12 | 10.00% | 1 | 0.83% | 13 | 10.83% | 0.526 |

tozobactam, cefoperazone + sulbactam, amikacin & meropenem.

The correlation in the previous study was not found to be significant ($P = 0.92$) for antibiotics: ampicillin, amikacin, cefoperazone with sulbactam, piperacillin with ctazobactam, ofloxacin, nitrofurantoin, and cefatazidime with clavulanic acid among biofilm and nonbiofilm producers⁵; whereas in our study, it is insignificant for imipenem & Nitrofurantoin ($P > 0.05$).

In the former study¹¹ among biofilm producers about 4.3%, 8.6%, 14.4%, 22.7%, 20.2%, 17.3%, and 6.0%, and among nonbiofilm producers: 19.3%, 6.4%, 19.3%, 21.5%, 3.2%, 3.2%, and 0%, were resistance to multiple drugs of 8, 9, 10, 11, 12, 13, and 14, respectively. In our study the biofilm producers *E.coli* are 0%, 12.5%,

20.19%, 15.38%, 16.35%, 16.35%, 12.5% and 6.73% while non producers *E.coli* are 18.75%, 12.5%, 0%, 0%, 0%, 0%, 0% and 0%, resistant to multiple drugs of 2, 3, 4, 5, 6, 7, 8 and 9 respectively.

It was observed that there was significant resistance pattern correlation between antibiotics (ampicillin, ceftriaxone, cefotaxime, ciprofloxacin, cotrimoxazole, norfloxacin, gentamicin, piperacilin tozobactam, cefoperazone + sulbactam, amikacin & meropenem) and biofilm producers. These antibiotics were not suggested for treating biofilm producing *E. coli* isolates. We recommended nitrofurantoin and imipenem for biofilm producing isolates.

In this study MDR isolates were found to be 90.83%, among which 95.41% were biofilm producers.

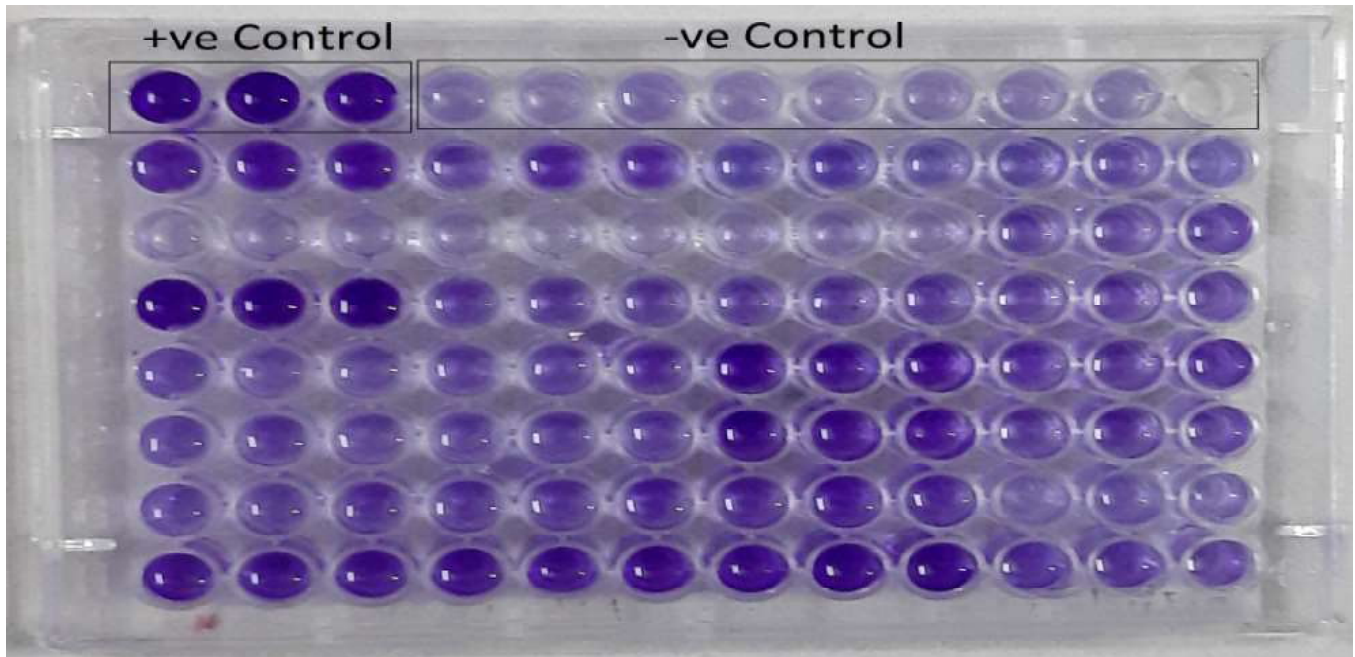


Fig. 1 : Biofilm formation

This is contrary to a study⁵ where 65% of the isolates reported were MDR and among these MDR isolates, 62 % were biofilm producers. This finding indicates that major percentage of biofilm producers are MDR, which are constantly allied to persistent infections and also fails to respond to treatment, instead it encourages the spread of antibiotic resistance amid nosocomial pathogens by mutation and by the exchange of antibiotic resistance encoded genes.

Conclusion

Biofilm producing *E.coli* are accountable for several unruly infections and are especially hard to eliminate due

to the likely acquisition of multidrug status. Severe antimicrobial resistance was detected in biofilm producing pathogens than non producers. Among the recommended antimicrobial therapies for biofilm producer CAUTIs, ampicillin and cephalosporins were the most resistive antibiotics, whereas Imipenem and nitrofurantoin were found as the most effective antibiotics against *E.coli* biofilm. Henceforth, an early identification of biofilm producers with subsequent detection of antibiotic resistivity pattern is obligatory, to advance the clinical management of CAUTIs once the patient needs an intensive care.

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