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**Original Research Article**

# Biofilm production and its correlation with antibiogram among clinical isolates of uropathogenic *Escherichia coli*

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## ABSTRACT

**Background:** Urinary tract infection (UTI) is one of the commonest health problem encountered in clinical practice. The biofilms play major role in decreasing the susceptibility to the antimicrobial agents. The present study aimed at isolation and identification of *Escherichia coli* from cases of urinary tract infection, and to find out production of biofilms by these isolates and to correlate it with antimicrobial resistance.
**Methods:** This study was a cross sectional study. Urine specimen from patients suspected of UTIs were collected and processed by standard operative procedure, antibiotic susceptibility test was done by Kirby Bauer disc diffusion method. Biofilm production in isolates was done by Tissue culture plate (TCP) method, Christensen's test tube (CTT) method, and Congo red agar (CRA) method.
**Results:** Out of the total of 250 isolates 191 (76%) showed biofilm positive by TCP method, 180 (72%) showed biofilm positive by CTT method, 161 (64%) showed biofilm positive by CRA method. 90% of total extended spectrum beta lactamase (ESBL) producing *Escherichia coli* were biofilm producers. There is more resistance for each and every antibiotic among biofilm producers in comparision to biofilm non producers.
**Conclusions:** The microbial biofilms may pose a public health problem, as the microorganisms in the biofilms are difficult to be treated with antimicrobial agents.

**Keywords:** Antibiotic resistance, Biofilm, *Escherichia coli*, Uropathogen

## INTRODUCTION

Urinary tract infection (UTI) is one of the commonest health problem encountered in clinical practice. *Escherichia coli* is the most frequently isolated microorganism in UTIs causing more than 80% of infections. Antimicrobial resistance has been recognized as an emerging worldwide problem. Many of the causative microorganisms tend to respond to the urinary tract environment by biofilm formation producing chronic and often intractable infection.[1] Biofilm is an aggregate of microorganisms in which cells are stuck to each other on to a surface and produce matrix of extracellular polymeric substance. Biofilm formation is regulated by the expression of polysaccharide intracellular adhesion molecule which mediates cell to cell adhesion and is a product of icaADBC genes.[2] Microbial biofilms are considered as the major problem in catheterized patients because they cause chronic infections which are difficult to treat, lead to longer hospitalization time, and can result in much higher treatment costs.[3] Biofilm development can be divided into three distinct stages: attachment of cells to a surface, growth of the cells into a sessile biofilm colony, and detachment of cells from the colony into the surrounding medium. The initial, transient attachment is reinforced by host- and tissue-specific adhesions that are located on the bacterial cell surface or on cellular appendages such as pili and fimbriae.[4] This results in the irreversible attachment of the bacterial cell to the surface. The second stage of

biofilm development involves the multiplication of bacteria on the surface and the concomitant synthesis of an extracellular polymeric matrix. The matrix holds the bacterial cells together in a mass and firmly attaches the bacterial mass to the underlying surface.[5] The final stage of biofilm development is the detachment of cells from the biofilm colony and their dispersal into the environment. This is an essential stage of the biofilm life cycle that contributes to biological dispersal, bacterial survival, and disease transmission.[6] The biofilms play major role in decreasing the susceptibility to the antimicrobial agents; as the proximity of cells within a biofilm can facilitate a plasmid exchange and hence enhance the spread of antimicrobial resistance. Keeping all these points in mind the aims and objects of present study are isolation and identification of *Escherichia coli* from cases of urinary tract infection, to find out production of biofilms by these isolates and to correlate it with antimicrobial resistance.

## METHODS

A Prospective study was carried out in the department of Microbiology of a tertiary care hospital from the period of November 2015 to October 2016. This was a cross sectional type of study. Institutional Ethics Committee clearance was obtained for the study. Urine specimen from patients suspected of UTIs were collected and inoculated onto Blood agar and MacConkey agar (Himedia laboratories, Mumbai) and incubated at 37ºC for 24 hours. A specimen was considered positive for UTI if the bacterial colony count is $>10^5$ cfu/ml. They were further processed for identification following standard operative procedures.[7] Antibiotic susceptibility test was performed by Kirby Bauer's disc diffusion method using Muller Hinton Agar (Himedia laboratories, Mumbai) as per Clinical and Laboratory Standards Institute (CLSI) guidelines and susceptibility pattern was noted.[8] The following antibiotic discs (Himedia laboratories, Mumbai) (drug concentrations in µg) were used: ampicillin (10µg), amikacin (30µg), gentamicin (10µg), ciprofloxacin (5µg), cotrimoxazole (25µg), nitrofurantoin (300µg), amoxiclav (20/10µg), piperacillin/tazobactam (100/10µg),ceftriaxone (30µg), ceftazidime (30µg), ceftazidime/clavulanic acid (30µg/10µg), meropenem (10µg).

Confirmatory test of ESBL Isolates considered potential ESBL producers by initial screening were emulsified with nutrient broth to adjust the inoculum density equal to that of 0.5 Mac Farland turbidity standards. Combination Disk test (CDT), as recommended by the CLSI, was performed in all isolates 1presumed to be ESBL producers. In this test, Ceftazidime (30 µg) disks alone and in combination with clavulanic acid (Ceftazidime + clavulanic Acid, 30/10 µg) disks, were applied onto a plate of Mueller Hinton Agar (MHA) which was inoculated with the test strain and then incubated in ambient air for 16-18 h of incubation at 35±2°C. Isolate that showed increase of ≥5 mm in the zone of inhibition of the combination discs in comparison to that of the Ceftazidime disk alone was considered an ESBL producer.[9]

Assessment of biofilm production by following three methods

***Tissue culture plate (TCP) method***

This quantitative test described by Christensen et al is considered the gold-standard method for biofilm detection.[10] Organisms isolated from fresh agar plates were inoculated in 10 mL of trypticase soy broth (Himedia laboratories, Mumbai) with 1% glucose. Broths were incubated at 37ºC for 24 h. The cultures were then diluted 1:100 with fresh medium. Individual wells of sterile 96 wellflat bottom tissue culture plates were filled with 0.2ml aliquots of the diluted cultures and only broth served as control to check sterility and non specific binding of media. The control organisms were also incubated, diluted and added to tissue culture plate. The plates were incubated at 37ºC for 24 h. After incubation, contents of each well were removed by gentle tapping. The wells were washed with 0.2 mL of phosphate buffer saline (pH 7.3) (Himedia laboratories, Mumbai) three times to remove free floating planktonic bacteria. Biofilm formed by bacteria adherent to the wells were fixed by methanol for 15 min and stained by crystal violet (0.1%). Excess stain was removed by using deionized water and plates were kept for drying. Optical density (OD) of stained adherent biofilm was obtained by using micro ELISA autoreader (Multiscan microplate reader, Labsystems Diagnostics, Tiilitie 3, Vantaa, Finland) at wavelength 620 nm. The experiment was performed in triplicate and repeated three times. The interpretation of biofilm production was done as follows.

*Calculation of cut off value*

The cut-off value optical density (OD) was calculated as 3 × standard deviation (SD) above the mean OD of 10 blank tissue culture plate wells stained by the above described procedure.

Mean OD = 0.055,

Standard deviation (SD) = 0.012

Therefore, cut off value = Mean + 3SD = 0.055+3(0.012) = 0.091 OD

*Interpretation of results*

- Non biofilm producers (non adherent)- OD value is less than the cut off value i.e. 0.091.
- Moderate biofilm producers (weakly adherent)- OD value is greater than 0.091 but lesser than or equal to 0.182.
- Strong biofilm producers (strongly adherent)- OD value is 2 × cut off value i.e. greater than 0.182.

***Christensen's test tube (CTT) method***

Described by Christensen et al this is a qualitative method for biofilm detection.[11] A loopful of test organisms was inoculated in 10 mL of trypticase soy broth (Himedia laboratories, Mumbai) with 1% glucose in test tubes. The tubes were incubated at 37ºC for 24 h. After incubation, tubes were decanted and washed with phosphate buffer saline (pH 7.3) (Himedia laboratories, Mumbai) and dried. Tubes were then stained with crystal violet (0.1%). Excess stain was washed with deionized water. Tubes were dried in inverted position. The scoring for tube method was done according to the results of the control strains. Biofilm formation was considered positive when a visible film lined the wall and the bottom of the tube. The amount of biofilm formed was scored as 1-none, 2-moderate and 3-high/strong. The experiment was performed in triplicate and repeated three times.

***Congo red agar (CRA) method***

Freeman et al have described a simple qualitative method to detect biofilm production by using Congo Red Agar (CRA) medium.[12] CRA medium was prepared with brain heart infusion broth 37 g/L, sucrose 50 g/L, agar No. 1 10 g/L and Congo Red indicator 8 g/L (Himedia laboratories, Mumbai). First Congo Red stain was prepared as a concentrated aqueous solution and autoclaved (121ºC for 15 minutes) separately from the other medium constituents. Then it was added to the autoclaved brain heart infusion agar with sucrose at 55ºC.5 CRA plates were inoculated with test organisms and incubated at 37ºC for 24 h aerobically. Black colonies with a dry crystalline consistency indicated strong biofilm production, brown colonies indicate moderate biofilm production while pink colonies indicate no biofilm production. The experiment was performed in triplicate and repeated three times.

***Quality control***

Quality control was maintained in all the steps and the tests was also carried out with control strains. The following ATCC (American Type Culture Collection) strains like *Staphylococcus aureus* ATCC 25923, and positive biofilm producer *Pseudomonas aeruginosa* ATCC 27853 were used control strains.

Statistical analysis was done by simple percentage method.

## RESULTS

A total of 1800 urine samples were processed during the study period, out of that approximately 25%(450) samples showed significant growth $>10^5$ CFU/mm$^3$. *Escherichia coli* was isolated from 250 (55.5% of growth positive samples) samples. The majority (64%) of the positive cases were females while the remaining (36%) were males. The frequency of *Escherichia coli* positive urine cultures were highest among the age group 41-50 years (21.7%) followed by 31-40 years (16.4%) as shown in Figure 1.

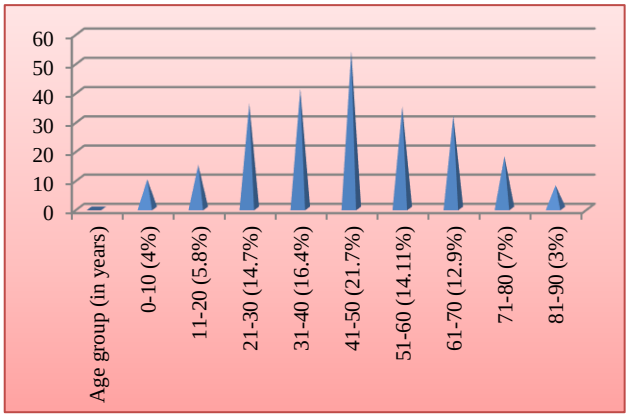


**Figure 1: Age wise distribution of urinary tract infections.**

Out of 250 *Escherichia coli* 143 (57%) were from wards, 97 (39%) from OPD and 10 (4%) from ICU. High level of drug resistance was seen in *E. coli* isolates. Among 250 *E. coli* isolated, highest sensitivity was found for amikacin (93.6%), nitrofurantoin (90.8%) and meropenem (88%) and gentamicin (75.2%) followed by cotrimoxazole (50%), piperacillin/tazobactum (40%), ciprofloxacin (23.2%), ceftazidime/clavulanic acid (23.2%) and ceftriaxone (21.2%).

**Table 1: Antibiotic sensitivity pattern of *Escherichia coli* isolates.**

| Name of the antibiotic | Number of sensitive isolates (%sensitivity) | Number of resistant isolates (% resistance) |
|---|---|---|
| Ampicillin | 25 (10%) | 225 (90%) |
| Amoxycillin/Clavulanic acid | 23 (9.2%) | 227 (90.8%) |
| Ciprofloxacin | 58 (23.2%) | 192 (76.8%) |
| Cotrimoxazole | 125 (50%) | 125 (50%) |
| Gentamicin | 188 (75.2%) | 62 (24.8%) |
| Amikacin | 234 (93.6%) | 16 (6.4%) |
| Nitrofurantoin | 227 (90.8%) | 23 (9.2%) |
| Meropenem | 220 (88%) | 30 (12%) |
| Ceftazidime | 13 (5.2%) | 237 (94.8%) |
| Ceftazidime/Clavulanic acid | 58 (23.2%) | 192 (76.8%) |
| Piperacillin/Tazobactum | 100 (40%) | 150 (60%) |
| Ceftriaxone | 53 (21.2%) | 197 (78.8%) |

Very few isolates (10%) were sensitive to ampicillin, amoxicillin/clavulanic acid (9.2%) and ceftazidime (5.2%) (Table 1). Out of the total of 250 isolates 191 (76%) showed biofilm positive by Tissue culture plate method 88 (35%) strong, 103 (41%) moderate and 59 (24%) none biofilm producers, 180 (72%) showed biofilm positive by CTT method 80 (32%) strong, 100

(42%) moderate and 70 (28%) none biofilm producers, 161 (64%) showed biofilm positive by CRA method, 73 (29%) strong, 88 (35%) moderate and 89 (36%) none biofilm producers (Table 2).

**Table 2: Comparison of biofilm production by three methods.**

| Total no. of isolates | Biofilm | TCP | CTT | CRA |
|---|---|---|---|---|
| 250 | Strong | 88 (35%) | 80 (32%) | 73 (29%) |
| | Moderate | 103 (41%) | 100 (40%) | 88 (35%) |
| | Weak | 59 (24%) | 70 (28%) | 89 (36%) |

All the isolates from ICU were found to be biofilm positive, while from wards and OPD only 83% and 65% isolates respectively are biofilm positive (Figure 2).

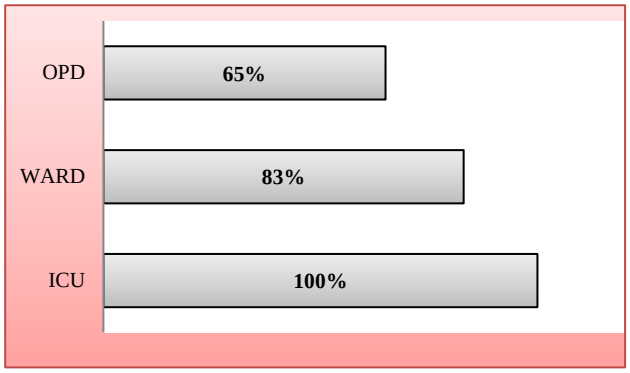


**Figure 2: Prevalence of biofilm formation in isolates from OPD, Wards and ICU.**

There is a total of 45 (18%) ESBL producer *Escherichia coli*. Out of 45, 40 (90%) isolates were biofilm producing and 5 (10%) were non biofilm producing while 143 (70%) isolates out of remaining 205 non ESBL producers isolates were biofilm producing and 61 (30%) were non biofilm producing (Figure 3).

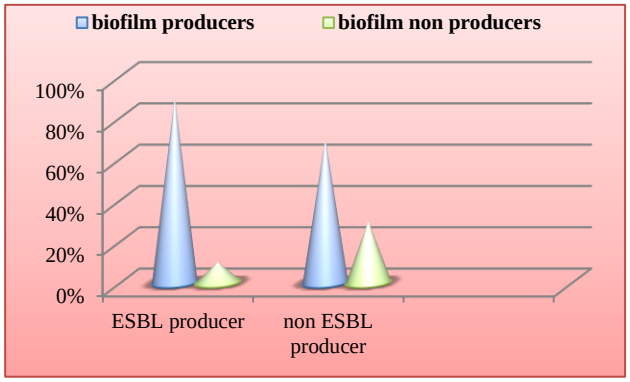


**Figure 3: Comparison of biofilm production in ESBL producer and non ESBL producers.**

There is more resistance for each and every antibiotic among biofilm producers in comparison to biofilm non producers (Figure 4).

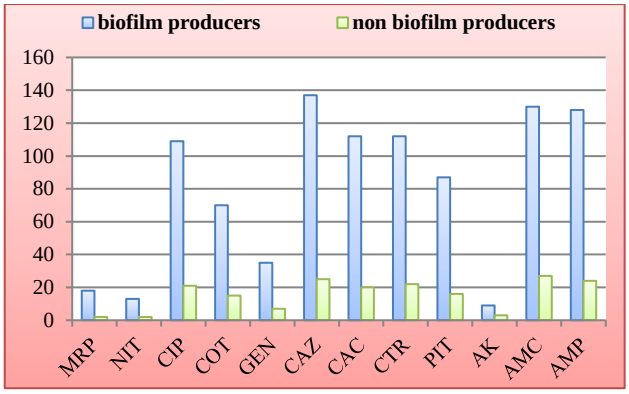


**Figure 4: Comparison of antibiotic resistance pattern between biofilm producers and non biofilm producers.**

## DISCUSSION

Biofilms are estimated to be responsible for over 65% of nosocomial infections and 80% of all microbial infections. Biofilm producing bacteria are responsible for many recalcitrant infections and are notoriously difficult to eradicate. They exhibit resistance to antibiotics by various methods like restricted penetration of antibiotic into biofilms, decreased growth rate and expression of resistance genes. In present study higher prevalence of UTI was seen in females 160 (64%) as compared to the males 90 (36%), thus showing a female predominance. Kamat US et al in their study noted females are more prone to develop UTIs, probably due to their anatomical physiological changes like short urethra, its proximity to the anus, dilatation of the urethra and the stasis urine during pregnancy.[13] In present study UTI was found frequently between 21-70 years which is similar to study done by Poovendran et al where it is 21-60 years and Iqbal et al where it is 15-50 years.[1,14] The high antibiotic resistance rates in *E. coli* causing UTI found by us was quite alarming although not entirely surprising. Out of the 250 isolates, 45(18%) were ESBL producers which is similar to a study done by Hong et al (18.3%) and lower than a study done by Parajuli et al in children (38.9%).[15,16] In this study high rates of resistance were found with ampicillin (90%), amoxicillin/clavulanate (90.8%), ceftazidime (94.8%), ceftriaxone (78.8%), ciprofloxacin and ceftazidime/clavulanic acid (76.8%) each, whereas resistance rate is low for amikacin (6.4%), nitrofurantoin (9.2%) and meropenem (12%), while in a study done by Mandal et al resistance for ampicillin (80.6%), ceftazidime (57.1%), ceftriaxone (60.5%), ciprofloxacin (70.04%) and by Bijapur et al high rates of resistance were found with ampicillin (91.66%), ciprofloxacin (75%), cotrimoxazole (71.87%), amoxycillin/clavulanate (63.54%) however, imipenem (0% resistance), meropenem (0%), piperacillin-tazobactam (15.62%), amikacin (5.2%) and nitrofurantoin (26.04%) appeared to have retained greater activity.[17,18] A low degree of resistance to amikacin (6.5%) and gentamicin (24.8%) was found which is in concordance with Sasirekha et al who found amikacin and gentamicin

resistance 12.5% and 31.3% respectively.[19] Oral nitrofurantoin , though has resistance rate of 10%, was found to be more effective in treatment of UTI and the findings are in agreement with similar surveillance studies by Sasirekha et al.[19]

In this study, TCP method detected 76% isolates as biofilm positive while CTT and CRA detected 72% and 64% respectively. According to Deka et al TCP Method detected 83%, Tube method detected 57% isolates whereas CRA detected 20% as biofilm producer whereas Mathur et al also showed that TCP method detected 53.9%, and CTT detected 57% as biofilm producers.[20,21] TCP detected 76% as biofilm producers and 24% as non biofilm producers while CTT detected 72% isolates as biofilm producers and 28% as non-biofilm producers. If we correlate CTT with TCP for identifying strong biofilm producers, then it was difficult to differentiate between moderate, weak and non-biofilm producers due to the change ability in the results detected by different observers. According to the previous studies, CTT cannot be suggested as general screening test to identify biofilm producing isolates. Moreover, we have performed the TCP method by addition of 1% glucose in trypticase soy broth. Addition of sugar helps in biofilm formation as reported by studies conducted by Mathur et al.[21]

In another study, Ruzicka et al noted that out of 147 isolates of *S. epidermidis*, CTT detected biofilm formation in 79 (53.7%) and CRA detected in 64 (43.5%) isolates, they showed that CTT is better for biofilm detection than CRA.[22] Baqai et al tested CTT to detect biofilm formation among uropathogens. According to their results, 75% of the isolates exhibited biofilm formation, with the CRA method, 11 were found to be biofilm producing bacteria and 99 as non-biofilm producers.[23] Knobloch et al did not recommend the CRA method for biofilm detection in their study, out of 128 isolates of *S. aureus*, CRA detected only 3.8% as biofilm producers as compared to TCP which detected 57.1% as biofilm producing bacteria.[24] In current study 100% isolates from ICU patients were biofilm positive, while 83% and 65% from ward and OPD respectively. The higher percentage in ICU and wards may be due to the severity of the underlying illnesses and iatrogenic factors related to the high frequency of invasive procedures needed for the monitoring and treatment. The ESBL producers were found to be more biofilm producing in comparision to non ESBL producers and the findings are in agreement with study done by Vasanthi et al.[2] In this study we have compared the antibiotic resistance between biofilm producers and non biofilm producers and found out that antibiotic resistance is much more higher for each and every antibiotic in case of biofilm producers in comparision to non biofilm producers, which is due to metabolically inactive persister cells. Present findings are in agreement with Deotale et al in a study of correlation between biofilm formation and highly drug resistant uropathogens (hdru).[4] According to Deotale et al 19 out of 30 biofilm producing isolates 20 (66.6%) were Highly Drug Resistance Uropathogens, and amongst 22 HDRU, 20 (90%) were biofilm producers. Sanchez et al also observed that strains capable of forming biofilms were more frequently observed to be an MDR phenotype.[25] Similar studies done by Poovendran et alconcluded that antibiotic resistance is higher in biofilm producing isolates in comparision to non biofilm producing isolates.[1]

## CONCLUSION

The microbial biofilms may pose a public health problem, as the microorganisms in the biofilms are difficult to treat with antimicrobial agents. Biofilm formation is closely related with the resistance of *Escherichia coli* towards the antimicrobial drugs and also it increases the chronicity of UTI. Tissue culture plate method is the gold standard for detection of biofilm formation. The future goal is to identify molecular targets of biofilm bacteria as well as the urinary components that are involved in biofilm for¬mation. Specifically, a better understanding of the genes responsible for biofilm formation in bacterial infections will provide the biological framework necessary for studying biofilms.

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