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In vitro ANALYSIS OF BIOFILM FORMATION AND ANTIBIOTIC RESISTANCE OF UROPATHOGENIC *Escherichia coli*

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ABSTRACT

Urinary tract infections are an exceedingly common worldwide problem, caused mostly by Gram-negative bacteria, especially *Escherichia coli*. Microbial biofilms are considered a serious public health problem. The potential of uropathogenic *E. coli* (UPEC) to produce biofilm was explored in the present study. The Congo red agar, tube- and tissue culture plate methods were used to evaluate the formation of biofilm by *E. coli* isolates. Of the 155 isolates, 101, 106 and 90 isolates were positive for these three methods, respectively. Subsequently, the sensitivity of *E. coli* isolates to antimicrobial agents (amikacin, cefepime, cefixime, cefotaxime, cefpodoxime, ceftazidime, ceftriaxone, ciprofloxacin, nitrofurantoin, gentamycin, nalidixic acid, ofloxacin and pefloxacin) was tested. There was no difference in the rate of biofilm detection between Congo red agar method and tube method. The antibiotic sensitivity test revealed that the biofilm-producing isolates were multi-drug resistant. The study emphasized the necessity for developing alternative therapeutic approaches to overcome multi-drug resistance arising from biofilm formation of UPEC.

Keywords: Antibiotic resistance, biofilm, uropathogenic *Escherichia coli*, urinary tract infection

INTRODUCTION

A microbial biofilm is a consortium of microbes enclosed within a polymer matrix produced by them. These can be a consortium of either a single species or multiple species which coexist in a suitable environment. The adherence of biofilm to the surfaces depends on several abiotic factors like surface charge, surface wettability, roughness, topography, stiffness, etc. (Schmalz *et al.*, 2021). The other factors that influence the biofilm adherence are temperature, osmolarity, concentration of ferrous iron ions, nutrient availability and quality, the materials on which biofilms are formed, and ambient acidity (Zheng *et al.*, 2021). The ability of biofilm production by a particular bacterium is determined by its adherence potential to a micro-titre plate alongside electron microscopy which gives the exact structure of biofilm *(*Novais *et al.*, 2013). The National Institutes of Health (NIH) has reported that the microbial and chronic infections associated with biofilm formation constitute 65 and 80%, respectively (Jamal *et al.*, 2018). Presumably, biofilm production is expected with enhanced urological infections and treatment repercussions (Javed *et al.*, 2020).

Urinary tract infections (UTI) are one of the significant reasons for illness and a potential cause for mortality in some cases such as pyelonephritis and urosepsis. Amongst the etiological agents responsible for the condition, the occurrence of *Escherichia coli* is relatively higher (80%) in community-acquired infections and 50% in hospital-acquired infections (Tajbakhsh *et al.*, 2016). The ability of UPEC to produce micro-colonies in uroepithelium is referred to as biofilm. This

phenomenon is of prominence due to its antibiotic resistance abilities, including enhanced virulence (Katongole *et al.,* 2020). Urolithiasis is a significant risk factor in the patients with recurring UTI. There is a distinct association between UTI and kidney stones. Some studies have indicated that *E. coli* activates sialidase which helps the transversion of uromucoid into a matrix rich in minerals whose outcome may be the reason for renal stone formation (Cetin *et al.,* 2020). Bacterial biofilms in urinary tract are capable of contributing towards recurrent UTI, chronic cystitis, prostatitis and relapses.

Drug resistance in bacteria is a potential problem in medicine, leading to increased treatment duration and potential mortality. The over- and under-use of antibiotics contribute to the development of resistance, as well as leads to the transfer of genetic material conferring resistance. Common bacterial resistance strategies include changes in drug target sites, increased cell wall permeability, antibiotic inactivation, and efflux pumps (Muhammad *et al.,* 2021). The drug resistance in bacteria might be due to the extracellular matrix of biofilm which prevents antimicrobials, hydroxyl radicals, and superoxide anions (Bhando *et al*., 2019). Serum resistance and biofilm formation are profoundly related with 78% of the isolates exhibiting both the phenomenon (Mittal *et al.,* 2015). Uropathogenic *E. coli* strains also exhibit higher serum resistance than commensal strains from the gastrointestinal tract (Zhou *et al.*, 2015). Biofilm formation and multidrug resistance were found correlated with 80% of the strains showing both properties (Subramaniam *et al.*, 2012). In *P. aeruginosa,* biofilm formation was found associated with the presence of biofilm-associated genes pelA and pslA, and increased resistance to antibiotics (Goudarztalejerdi *et al., 2022*). Similarly, in *E. coli*, the strains with stronger biofilm formation showed a higher proportion of multidrug-resistant isolates (Qian *et al.*, 2022).

Biofilm-associated microbes behave in different way in comparison to the planktonic organisms, including differences in growth rate and resistance to antimicrobial treatments (Bekő *et al.,* 2022). These differences pose a significant public health problem, as biofilms can lead to the increased biomass and enhanced antimicrobial resistance, making them more difficult to treat (Kwok *et al.,* 2022). Thus there is urgent need to study biofilm profile and their ability to evade antibiotic sensitivity. The present study aims to screen the ability of uropathogenic *E. coli* (UPEC) to produce biofilm under *in vitro* conditions and its antibiogram.

MATERIALS AND METHODS

The present study was conducted in the Department of Microbiology & Biotechnology, Presidency College, Chennai (India). The study involved fresh culture 155 uropathogenic *E. coli* isolates, previously isolated from urinary tract infected patients attending the hospital during January-December, 2021. A loopful of inoculum was transferred to fresh nutrient broth and incubated for 24 h at 37°C. The culture was diluted with fresh broth to achieve optical densities corresponding to 0.5 McFarland standard (Scott and Sutton, 2011).

Detection of biofilm formation

The isolates were tested using three methods: tube method, Congo red agar method, and tissue culture plate method (Sutton. 2019). The experiment was conducted in a completely randomized design with each treatment replicated three times. Tissue culture plate method was used as a standard for detecting biofilm formation. The data was analysed statistically to determine significant differences between the methods (Sutton, 2019) and their ability to accurately detect biofilm production.

Congo red agar method: Congo red agar (CRA) method was used to quantify biofilm production in *E. coli* isolates. Brain heart infusion broth with Congo red, supplemented with 5% sucrose, was inoculated with the culture and incubated aerobically for 24-48 h at 37°C. Black colonies with dry crystalline consistency indicated a positive result while the colonies remaining pink revealed weak biofilm production and dark colonies with no crystalline texture indicated indeterminate results (Karigoudar *et al.,* 2019).

Glass tube method: A tube containing 1 mL trypticase soy broth with 1% glucose was inoculated with *E. coli* and incubated at 37°C for 24 h. Then, the medium was removed and washed with phosphate buffer saline (PBS) (pH 7.3) and dried. The tubes were stained with 1.5 mL of 0.1% crystal violet and the excessive stain removed by washing with deionized water. Tubes were then dried in inverted position and observed for biofilm formation (Chen *et al.,* 2018). The visible film lining the wall and bottom of tubes indicated positive test for biofilm formation while the ring formation at liquid interface indicated negative test for biofilm formation.

Tissue culture plate (TCP) method: The tissue culture plate assay protocol delineated by O'Toole (2011) was followed. The isolates of *E. coli* were inoculated in 10 mL trypticase soy broth (TSB) with 0.25% glucose and incubated overnight at 37°C. The cultures were diluted in 1:100 ratio, and 200 µL diluted cultures were inoculated into 96-well polystyrene micro-titre plates. After 48 h incubation, the plates were washed thrice with 300 µL distilled water. Subsequently, the plates were stained with 200 µL of 1% crystal violet for 10 min. The plates were gently washed twice with distilled water to remove excessive crystal violet. Finally, 250 µL of 95% ethanol was added to the plates and absorbance of destained solution measured at 570 nm in an Elisa reader (Biotek EL x 800, USA). A well with sterile TSB served as control. Optical density measurement revealed that positive biofilm formation ranged between OD value 0.200 and 0.299, while negative biofilm formations were less than 0.100 OD.

Antibiotic susceptibility test (AST)

Kirby-Bauer disc diffusion method was followed to measure antibiotic susceptibility of isolates (Clinical Laboratory Standards Institute, 2016). Antibiotic discs used in the study were: amikacin (30 mg), cefepime (30 mg), cefixime (5 mg), cefotaxime (30 mg), cefpodoxime (10 mg), ceftazidime (30 mg), ceftriaxone (30 mg), ciprofloxacin (5 mg), nitrofurantoin (30 mg), gentamycin (10 mg), nalidixic acid (30 mg), ofloxacin (5 mg) and pefloxacin (30 mg) (all procured from Hi-Media, India) were utilized for performing antibiotic susceptibility test. These antibiotic discs were placed in 2 Mueller Hinton agar plates. The plates were incubated at 37˚C for 24 h, and the diameter of each zone was measured. The zones of inhibition were interpreted using the Clinical Laboratory Standards Institute (CLSI), 2016. The tests were carried out in triplicate.

Statistical analyses

The data was statistically analysed by using IBM Statistical Package for the Social Sciences for Windows, version 26.0 (IBM Corp., New York, USA). The statistical analysis with respect to χ^2, P-value, and correlation coefficient was performed by using Instat Software. P value $\leq$ 0.05 was considered significant. TCP is deemed as a gold standard method for biofilm detection among the three test methods, and the other statistical parameters were calculated accordingly (Panda *et al.*, 2016).

RESULTS AND DISCUSSION

The 155 *E. coli* isolates were subjected to biofilm production by three methods. In Congo red agar (CRA) method 101 isolates formed biofilm while 54 isolates produced either weak biofilm or were indeterminable (Table 1). Comparatively in tissue culture plate (TCP) and glass tube methods 106 and

Table 1: Biofilm and non-biofilm forming *E. coli* isolates (155) detected by different methods

Methods followed	Biofilm producers (No.)	Non-biofilm producers (No.)
Congo red agar plate (CRA)	101 (65.16%)	54 (34.83%)
Tube method (TM)	106 (68.39%)	49 (31.61%)
Tissue culture plate (TCP)	90 (58.07%)	65 (41.93%)

Table 2: Statistical evaluation of Tube and CRA methods for detection of biofilm formation in clinical isolates (n = 155) taking tissue culture plate method as the gold standard

Screening methods	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
Tube method	74.4	40.0	63.2	53.1
Congo red agar method	74.4	46.2	65.7	56.6

90 isolates, respectively, produced biofilm. The biofilm producing isolates in our study were higher than those of Siddhiqui *et al*. (2018) who using CRA assay on 112 *E. coli* isolates found 5 (4.5%) strong biofilm producers and 11 (9.8%) moderate producers. Further, they found that 20 (17.8%) and 37 (33.0%) isolates developed weak and moderate biofilm by using tissue culture plate method. However, Dash *et al.* (2018) by using tissue culture plate approach found 54.4% uropathogenic isolates as biofilm producers.

Statistical analysis of data was performed to assess the accuracy, sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of tube adherence method and CRA, with the TCP method serving as the gold standard (Table 2). The tube adherence method showed sensitivity and specificity (74.4 and 40.0%) approximately equal to CRA method (74.4 and 46.2%). On the other hand, the NPV and PPV were nearly identical. The Cramer's V coefficient indicated a moderate association between CRA and TCP methods, with p-value less than 0.05. However, a weak association existed between tube and TCP methods, with a p-value greater than 0.05. The CRA and TM assays can easily identify biofilm-producing strains, but distinguishing weak and negative isolates is a challenging task, so affecting their sensitivity and specificity (Mohsenzadeh *et al.*, 2021). The study revealed that TM and CRA have higher sensitivity but also have high rate of false-positives, indicating they may not be suitable for general screening of biofilm producers. TCP method appears more reliable for rapid screening of biofilm producers and their early detection can reduce untreatable infections. Tube and CRA methods can be useful in resource-poor settings.

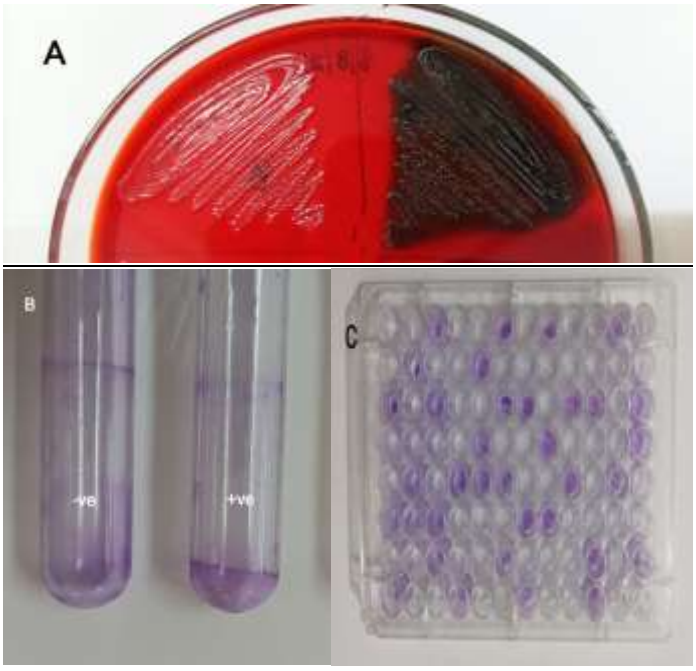

Fig. I: *E. coli* isolates showing positive and negative results A - Congo red agar - black colonies indicates biofilm positive and red/pink colonies indicates biofilm negative, B - visible film lining the wall and bottom of the tube in tube method; C – Micro-titre plate showing biofilm production by TCA method

A total of 13 antibiotic discs were placed in Mueller Hinton agar plate for each *E. coli* isolate so as to assess whether they were biofilm producing or not. The study showed that all 155 *E. coli* isolates tested were resistant to cefixime, cefepime, and ceftazidime; while 150 isolates showed resistance to ceftriaxone and cefpodoxime. Further, 146 isolated were resistant to Cefotaxime, 135 isolates to nalidixic acid. Pertinently only 16% were resistant to gentamycin, 14% to pefloxacin and 5% to amikacin. The antibiogram pattern of *E. coli* isolates is shown in Fig. 2. Drug resistance in *E. coli* is a major medical concern for both outpatients and hospitalised patients. Biofilm

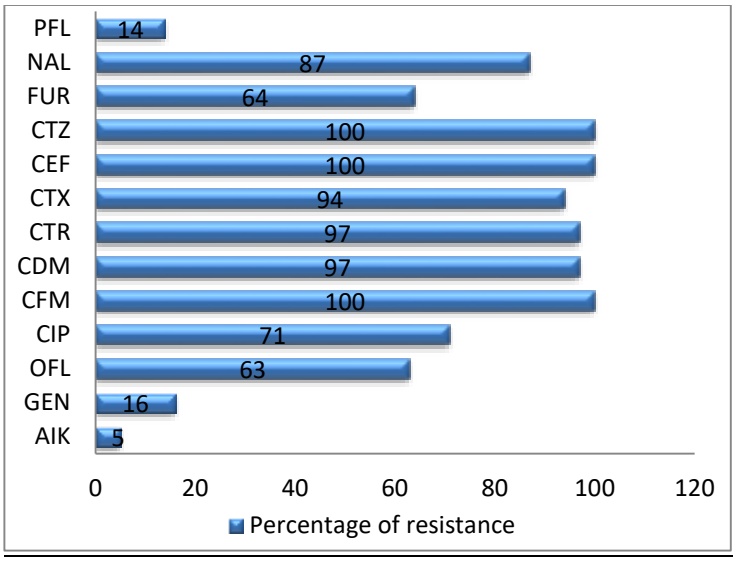

Fig. 2: Antibiotic susceptibility of *E. coli* isolates; AIK - amikacin, GEN - gentamycin, OFL - ofloxacin, CIP - ciprofloxacin, CFM - cefixime, CDM - cefpodoxime, CTR - Ccftriaxxone, CTX - Cefotaxime, CEF - Cefepime , CTZ - Ceftazidime, FUR - Nitrofurantoin, NAL - Nalidixic acid and PFL – Pefloxacin

formation contributes to bacterial antibiotic resistance by interfering with drug movement in their anatomical region. Further, biofilms induce transcriptional changes via quorum sensing mechanisms (Behzadi *et al.*, 2020). The level of antibiotic resistance varies with the stage of biofilm. Antibiotics work most effectively in reversible attachment step because bacteria are not connected themselves to the matrix. Due to this, they are at risk from the action of antibiotics and the immune system of the host (Risal *et al.*, 2018). In biofilm producers, poor antibiotic penetration, slow growth, adaptive stress responses, nutrient limitation, and the formation of persister cells are supposed to constitute a multi-layered defence (Dash *et al.*, 2018). It is suggested that around 100 to 1000 times more antibiotic resistance is found in microbial cells found in biofilms than in planktonic cells (Sharma *et al.*, 2019).

Nitrofurantoin, trimethoprim, and cefalexin were the antibiotics used as first choice against uropathogenic *E. coli* in women, while amoxicillin, trimethoprim/sulfamethoxazole, andamoxicillin/ clavulanic acid were used as second choice drugs, when the prescribed first-choice drugs were ineffective (Arafa *et al.*, 2022). UPEC biofilms were found precisely resistant to certain antibiotics. Beta-lactams such as cefpodoxime, ceftazidime, and aminoglycosides such as gentamycin and amikacin were ineffective (Ponnusamy *et al.*, 2012). Our results suggest that a significantly large number of urinary tract infection patients are at risk of developing multidrug resistance indirectly, as there are several correlations implicated between biofilm formation and drug resistance.

Cefixime, cefepime, and ceftazidime were the most resistant antibiotics, while gentamycin, pefloxacin, and amikacin were least resistant. The results suggest that biofilm producers of uropathogenic *E. coli* may have a higher likelihood of developing antibiotic resistance as compared to the non-producers. Understanding the specific antibiotics that are most and least resistant can help in framing treatment strategies for UPEC infections. Biofilms are a serious public health problem due to the increased resistance of constituent organisms to antimicrobial drugs and their ability to cause infection, especially in the patients implanted with medical devices (Haji, 2018). These study suggests that the ability of *E. coli* to produce biofilm varies depending upon the specific strain and the method used for assessment. The variation in biofilm production highlights the importance of monitoring this phenomenon so as to develop effective strategies for combating antibiotic resistance.

Conclusion: The study revealed the occurrence of biofilm producers and their antibiotic susceptibility pattern for UPEC isolates. It emphasized on the immediate need to explore alternative therapeutics so as to overcome multidrug resistance induced by biofilms in patients affected by UTI caused by UPEC. Further, this study highlights the necessity of maintaining a sterile environment in hospital premises.

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