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Study of Correlation between Antimicrobial Resistance and Biofilm Production among Uropathogenic *Escherichia Coli* Isolated from Patients Suspected of Urinary Tract Infection

Debabrata Barik[1], Ashisa Kumar Das[2], Dibya Prasana Mohanty[3], Susmita Kumari Sahu[4]

Abstract: ***Background & objectives: Escherichia coli is a primary causative agent of recurrent urogenital infections. Biofilm producing strains causes recurrent and chronic UTI and they exhibit multidrug resistance. Our study aims to estimate the prevalence of biofilm production in the isolated Uropathogenic Escherichia coli strainsand to correlate the association of biofilm production with their resistance pattern to commonly used antimicrobials. Materials and methods: A prospective cohort study was conducted from August-November 2021 in Department of Microbiology, MKCG MCH, Berhampur, Odisha that included urine samples of all suspected UTI patients. Specimens were inoculated on CLED agar plate, then incubated at 37ºC for 24 hours under aerobic conditions. By standard microbiological methods, the isolates were identified. Isolates of Escherichia coli with significant bacteriuria were processed for biofilm detection by Congo Red Agar method, Tube method and TCP method. Antimicrobial Susceptibility testing was performed by Kirby–Bauer disc diffusion method following recent CLSI guidelines. Results: Of the 102 Escherichia colistrains, 49 (48.03%) and 53 (51.96%) were from catheterized and Non-catheterized patients respectively. Biofilm production by CRA, TM, and TCP method were 61 (59.80%), 70 (68.62%), and 78 (76.47%) respectively. Biofilm producers showed maximum resistance to Cefotaxime, Levofloxacin and Amoxycillin-Clavulanic acid when compared to nonbiofilm producers. Conclusion: Biofilm producing Escherichia coli strains exhibits higher resistance to most of the commonly used antimicrobials than non producers.***

Keywords: Microbiology, Escherichia coli, Biofilm, Congo, red agar, Tube method, Tissue culture, plate method, Resistance Uropathogen

1. Introduction

Escherichia coli, is usually a harmless gut commensal in humans and other mammals. But it may develop into an intra-or extra-intestinal pathogen. Urinary tract infection (UTI) is one of the frequent causes of morbidity in the general population**. [1]** Catheterized patients are at an increased risk of developing bacteriuria [**2].** The majority of the patients with an indwelling urinary catheter for 30 days or longer are prone to develop bacteriuria [**3].** *Escherichia coli* is also a frequent colonizer of medical devices and the primary cause of recurrent urogenital infections accounting to about 70%–95% of UTIs. [**4, 5, 6, 7]** *Escherichia coli* is known to form intracellular bacterial communities with biofilm-like properties within the epithelium of urinary bladder [**8].** Biofilms are a congregation of microbial cells formed by bacterial species that are irreversibly associated with a surface and enclosed in a matrix of polysaccharide and protein material. This confers a number of advantages such as protection from antimicrobial agents, exchange of nutrients and exchange of genetic material. Biofilm producers exhibit an altered phenotype with respect to growth rate and gene transcription. Biofilm producing bacteria causes recurrent and chronic UTI there by contributing to longer stay in hospital, increased cost of treatment and difficult to treat as they exhibit multidrug resistance (MDR) **[9, 10]**. The prevalence of biofilm producing Uropathogenic *Escherichia coli (*UPEC) ranges from 60% to 70% [**1, 4, 8].**

2. Materials & Methods

This prospective study was carried out in the Department of Microbiology, M. K. C. G. Medical College and Hospital, Berhampur, Ganjam, for three months.

Inclusion criteria

Male and female patients of all age groups with symptoms of Urinary Tract Infection attending various outpatient departments or admitted in wards of hospital were included in the study.

However, repeat samples of the same patient and patients who were on antibiotic therapy or had history of antibiotic intake within one week prior to sample collection were excluded from the study.

Sample collection and processing

Under proper aseptic conditions, mid-stream urine sample of patients were collected in sterile containers. They were transported to the laboratory as soon as possible and processed immediately.

Urine samples were inoculated onto Cystine lactose electrolyte-deficient (CLED) medium and incubated at 37°C overnight. The isolates were identified on the basis of the colony morphology, Gram-staining and the standard biochemical tests. Isolates of *Escherichia coli* with significant bacteriuria were subjected to biofilm detection. Antimicrobial Susceptibility testing was performed by Kirby–Bauer disc diffusion method following recent CLSI guidelines. **[11]**

Detection of Biofilm production

Three methods were carried out for detection of biofilm production with Escherichia coli ATCC 25922 as the positive control –

Congo Red Agar Method (CRA),
Tube Method (TM) and
Tissue culture plate method (TCPM).

Congo Red Agar Method

The following reagents from Himedia labs were used:

Brain heart infusion broth (BHI) (37 g/l) with sucrose (50 g/l),

Agar No 1 (10g/l) and
Congo red (0.8 g/l).

Congo Red stain was prepared and autoclaved separately. Then it was added to the autoclaved brain heart infusion agar with sucrose at 55ºC and poured onto petri plates. These plates were inoculated with the test organisms and incubated at 37°C for 24 hours under aerobic conditions. Biofilm Producers were characterized by formation of Black colonies with dry and crystalline consistency. **[12, 13]**

Tube Method

A loopful of test organism (incubated overnight) was inoculated into glass tubes with 10ml of Trypticase soy broth with 1% glucose. These tubes were then incubated at 37°C for 24 hours aerobically. After incubation, the tubes were decanted and washed with phosphate buffer saline at pH 7.3 and dried, after which the tubes were stained with crystal violet (0.1%) for 15 minutes. The stain was decanted and the tubes were washed with de-ionised water and dried in inverted position. Biofilm formation was identified by a visible film lining the walls and the bottom of the tube. **[12, 14]** In some of the tubes, formation of a stained layer at the air-liquid interface was seen. However it was considered negative for biofilm formation.

Tissue Culture Plate Method

Fresh Isolates were inoculated in Trypticase Soy Broth and incubated for 24 h at 37°C, then diluted with fresh Trypticase Soya Broth to achieve 1 in 100 dilution.0.2 ml aliquots of the diluted cultures was poured using a pipette into each well of a sterile, polystyrene, 96 well-flat bottom tissue culture plate (TCP). For negative control only broth was used. The TCP was incubated for 18–24 h at 37°C. Then the content of each well was carefully removed by tapping the plates. Then wells were washed three times with 0.2 ml of PBS (pH 7.2). Wells were stained with crystal violet (0.1%). Excess stain was washed out with deionized water. The plate was then dried. In a biofilm producing strain, the wells are uniformly stained with crystal violet. The Optical density (OD) was measured with a ELISA auto reader at a wavelength of 570 nm (OD 570 nm). After repeating the experiment twice, the data was averaged. **[12, 15]**

3. Results

Out of the 102 *Escherichia coli* strains, 49 (48.03%) and 53 (51.96%) strains were from catheterized and non-catheterized patients, respectively. Of the 102 *E. coli* isolates subjected to biofilm production, Sixty-one (59.80%), Seventy (68.62%) and Seventy eight (76.47%) were positive for biofilm productions by Congo Red Agar (CRA), tube method (TM), and TCP method, respectively. Isolates from catheterized patients showed maximum biofilm production. Among the 49 (48.03%) catheterized patients, 33 (67.34%), 38 (77.55%) and 43 (87.75%) strains were biofilm producer by Congo Red Agar, Tube method, TCP method, respectively.

Among the non-catheterized patients, TCP method detected 35 (66.03%) biofilm producers, Tube Method detected 32 (60.37%) biofilm producers, and CRA detected 28 (52.83%) biofilm producers, respectively.

Overall 87.75% and 66.03 % of *E. coli* strains were biofilm producers from catheterized patients and non-catheterized patients.

The correlation of catheterization and biofilm production by different methods has been given in the following table.

	TCP	TM	CRA
Catheterized (49)	43 (87.75%)	38 (77.55%)	33 (67.34%)
Non-Catheterized (53)	35 (66.03%)	32 (60.37%)	28 (52.83%)
Total (n) =102	78 (76.47%)	70 (68.62%)	61 (59.80%)

A significant correlation was found between biofilm production and catheterization. Out of 102 *E. coli* strains, maximum number of isolates were from female patients 60 (58.8%) compared to male patients 42 (41.2%).

The age wise distributions of both male and female sexes are given in the table below.

Age wise details	
0-10	10
>10-20	06
>20-30	30
>30-40	14
>40-50	06
>50-60	24
>60	12
Total (n) = 102	

The following tables show overall resistance pattern of the isolated strains.

	Sensitive	Resistant	Total
Ampicillin-sulbactam	42	60 (58.82%)	102
Amoxycillin – Clavulanic acid	27	75 (73.52%)	102
Cotrimoxazole	43	59 (57.84%)	102
Gentamicin	83	39 (38.23%)	102
Levofloxacin	24	78 (76.47%)	102
Cefotaxime	15	87 (85.29%)	102
Cefepime	28	74 (72.54%)	102
Meropenem	96	16 (15.68%)	102

Nitrofurantoin	87	15 (14.70%)	102
Piperacillin-Tazobactam	78	44 (43.13%)	102

	Biofilm Producers (n=78)		Non-Producers (n=24)	
	Sensitive	Resistant	Sensitive	Resistant
Ampicillin-sulbactam	24	54 (69.23%)	18	06 (25%)
Amoxycillin – Clavulanic acid	08	70 (89.74%)	19	05 (20.83%)
Cotrimoxazole	23	55 (70.51%)	20	04 (16.66%)
Gentamicin	42	36 (46.15%)	21	03 (12.5%)
Levofloxacin	07	71 (91.02%)	17	07 (29.61%)
Cefotaxime	04	74 (94.87%)	11	13 (54.16%)
Cefepime	08	70 (89.74%)	20	04 (16.66%)
Meropenem	64	14 (17.94%)	22	02 (8.33%)
Nitrofurantoin	65	13 (16.66%)	22	02 (8.33%)
Piperacillin-Tazobactam	41	37 (47.43%)	17	07 (29.16%)

Among the biofilm producers, maximum resistance was seen to Cefotaxime (94.87%), Levofloxacin (91.02%), Cefepime, Amoxycillin-Clavulanic acid (89.74%), Co-trimoxozole (70.51%), Ampicillin-sulbactam (69.23%), Piperacillin-Tazobactam (47.43%), Gentamicin (46.15%). Minimal resistance was seen to Meropenem (17.94%), followed by Nitrofurantoin (16.66%).

	Non-Biofilm Producers (n=24)	
	Sensitive	Resistant
Ampicillin-sulbactam	18	06 (25%)
Amoxycillin – Clavulanic acid	19	05 (20.83%)
Cotrimoxazole	20	04 (16.66%)
Gentamicin	21	03 (12.5%)
Levofloxacin	17	07 (29.61%)
Cefotaxime	11	13 (54.16%)
Cefepime	20	04 (16.66%)
Meropenem	22	02 (8.33%)
Nitrofurantoin	22	02 (8.33%)
Piperacillin-Tazobactam	17	07 (29.16%)

Among the non-biofilm producers, maximum resistance was seen to Cefotaxime (54.16%), Levofloxacin (29.61%), Piperacillin-Tazobactam (29.16%), Ampicillin-Sulbactam (25%), Amoxyclav (20.83%) and, minimum resistance was seen to Meropenem and Nitrofurantoin (8.33%).

4. Discussion

Time and again multiple studies have been undertaken to prove the correlation between biofilm production and resistance to commonly used antibiotics.

In a study conducted by Saroj *et al.*, they found 69% of the isolates of *E. Coli* were biofilm producers by TM and TCP methods. **[16]** Whereas Sevanan *et al.*, found out 59.4% strains to be biofilm producers by Congo red agar method. **[8]** In another study conducted by Sharma *et al.*, 67.5% isolates of *E. Coli* showed biofilm production by TCP method. [**17]** In our study, biofilm production was seen in 59.80%, 68.62% and 76.47% of isolates by Congo Red Agar (CRA), tube method (TM), and TCP method, respectively.

Our study is in concordance with the studies undertaken by the above authors.

In their study, Saroj *et al.* categorized the study subjects into catheterized and noncatheterized ones. What they found out was among 67 isolates of *E. coli* from catheter - associated UTI patients, 89.5% isolates produced biofilm by all the three methods. Among symptomatic non-catheterized UTI patients, 56% were biofilm producers by TCP method, 48% by TM method, and 72% by CRA method.

In our study, we found out that among the 49 (48.03%) catheterized patients, 43 (87.75%) and 6 (12.25%) strains were biofilm and nonbiofilm producer by TCP method, respectively. In Tube method of biofilm detection, 38 (77.55%) and 11 (22.45%) were biofilm producers and non producers, respectively. By CRA method, 33 (67.34%) and 16 (32.66%) strains were biofilm producers as non producers respectively. Among the 53 (51.96%) non-catheterized subjects, TCP method detected 35 (66.03%) as biofilm producers and 18 (33.97%) as nonbiofilm producers. By TM, 32 (60.37%) and 21 (39.63%) were biofilm and nonbiofilm producers, respectively. By CRA, 28 (52.83%) and 25 (47.17%) were biofilm and non-biofilm producers, respectively.

These variations could be because of difference in strain number as well as due to testing of these *E. coli* isolates under different laboratory conditions.

Poovendran *et al.*, found out that all biofilm forming strains exhibited maximum resistance to amoxyclav (100%), followed by chloramphenicol (100%), gentamicin and cefotaxime (86% each), ceftazidime (84%), cotrimoxazole, and piperacillin with tazobactam (83% each), and amikacin (70%). Resistance to co-trimoxazole (83% vs. 53%), tetracycline (75% vs. 50%), and ampicillin (64% vs. 50%) were higher in biofilm producing strains than non-producers. Moreover biofilm producing isolates were resistant to multiple antibiotics. **[18**] The study by Sevanan *et al.* also showed that biofilm producing *Escherichia coli* isolates were more resistant to antibiotics than non-biofilm producing isolates. The resistant pattern of erythromycin, amikacin, co-trimoxazole, ampicillin, meropenem, chloramphenicol, tobramycin, and gentamicin were found to be in the order of 90.6 %, 71.9%, 65.6%, 59.3%, 56.3%, 56.3%, 53.1%, and 50.0%, respectively among biofilm producing isolates. Resistance was seen least with amoxicillin (37.5%) and cephalexin (18.8%).

In our study, the biofilm producers showed maximum resistance to Cefotaxime (94.87%) followed by Levofloxacin (91.02%) followed by Cefepime and Amoxycillin-Clavulanic acid (89.74%), Co-trimoxozole (70.51%), Ampicillin-sulbactam (69.23%), Piperacillin-Tazobactam (47.43%), Gentamicin (46.15%). Minimal resistance was seen to Meropenem (17.94%), followed by Nitrofurantoin (16.66%). Among the non-biofilm producers, maximum resistance was seen to Cefotaxime (54.16%), Levofloxacin (29.61%), Piperacillin-Tazobactam (29.16%), Ampicillin-Sulbactam (25%), Amoxyclav (20.83%) and minimum resistance was seen to Meropenem and Nitrofurantoin (8.33%).

This high rate of resistance among both biofilm and non-biofilm producers could be due to factors like

extended-spectrum β-lactamases (ESBLs), AmpC, beta-lactamases or carbapenemases.

From our findings we deduce that nitrofurantoin and meropenem are best effective against biofilm producing uropathogenic *Escherichia coli* strains.

5. Conclusion

Urinary Tract Infections are amongst the commonly encountered problems in clinical practice. Furthermore due to increased incidence of biofilm producing strains of bacteria, they have become very difficult to treat by exhibiting resistance to mulitiple antibiotics that leaves the clinician with limited resources. The focus should be on early identification of biofilm forming uropathogenic Escherichia coli so that effective control strategies and further management of these problematic UTIs can be planned. Unlike the conventional methods, which are time consuming, molecular methods can be opted for rapid diagnosis of these conditions. Tissue Culture Plate method is the most suitable and reliable method whereas CRA method and tube method can be employed for biofilm detection in resource constraint conditions.

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