Biofilm and multidrug resistance in uropathogenic *Escherichia coli*

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Context: *Escherichia coli* is known as causative agent of urinary tract infections (UTIs) tends to form microcolonies in mucosa lining of urinary bladder known as biofilm. These biofilms make the organism to resist the host immune response, more virulent and lead to the evolution of antibacterial drug resistance by enclosing them in an extracellular biochemical matrix.

Aims: This study was done to know the association of various virulence factors and biofilm production in uropathogenic *E. coli* (UPEC) and antibiotic susceptibility pattern.

Settings and design: This study was conducted in Pt. B.D. Sharma PGIMS, Rohtak, Haryana during a period of 1 year from January 2011 to December 2011.

Methods and material: Biofilm was detected by microtiter plate (MTP) method, and various virulence factors like hemolysin, hemagglutination, gelatinase, siderophore production, serum resistance, and hydrophobicity were detected. The antibiotic susceptibility testing was done by modified Kirby-Bauer disk diffusion and the disk diffusion method was used to confirm the ESBL, AmpC, MBL production by the UPEC statistical analysis used: The data were analyzed by using SPSS version 17.0. A two-sided *P*-value of less than or equal to 0.05 was considered to be significant.

Results: Biofilm production was found in 18 (13.5%) isolates, more commonly in females (two times). These isolates were found to be resistant to antibiotics common in use and were 100% MDR.

Conclusions: Biofilm production makes the organism to be more resistant to antibiotics and virulent as compared to non-biofilm producers.

Keywords: Uropathogenic *Escherichia coli*, Biofilm, Drug resistance, Virulence factors

Introduction

Urinary tract infection (UTI) represents one of the major nosocomial infections, commonly caused by *Escherichia coli*, which accounts for 90% of community acquired and 50% of hospital acquired UTI.¹ These *E. coli* isolates usually originate from patients' intestinal normal flora; however, when fecal *E. coli* colonizes the periurethral area and lead to UTI, these isolates are known as uropathogenic *E. coli* (UPEC).

These UPEC have several virulent determinants e.g. fimbriae 1, hemolysin, hydrophobicity, serum resistance, biofilm production, etc. which enables them to colonize the bladder mucosa and injure it, leading to inflammatory changes and overcome the host immune regulators.²

When the causative organism tends to colonize the bladder mucous membrane in microbial communities, it is known as biofilm and these microcolonies favor the long-term persistence of microbe in host tissue. These microcolonies are impermeable to

many antibiotics, leading to the evolution of multi-drug-resistant strains of microorganisms, which is responsible for relapses in untreatable UTI.

This study was completed in order to know the biofilm formation in UPEC, correlate it with other virulence factors, e.g. hemolysin, hemagglutination, hydrophobicity, serum resistance, siderophore, and gelatinase production and detect the antibiotic susceptibility with special reference to production of ESBL, AmpC, and MBL.

Subjects and Methods

The present study was conducted on 135 *E. coli* isolates recovered from UTI cases in the time period of 1 year from January to December 2011. Samples were selected randomly from both outdoor as well as indoor patients of all age groups (Table 1). Urine samples were processed immediately and *E. coli* isolates were identified by the standard microbiological procedures, as per standard protocols.³

Biofilm production was detected by microtiter plate (MTP) method,⁴ in which the ability of microorganisms to form biofilm on abiotic surfaces

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Table 1 Age wise distribution of UTI cases

Age groups (years)	Females	Males
0–10	4	2
11–20	5	5
21–30	37	10
31–40	10	10
41–50	5	6
51–60	6	10
61–70	5	14
71–80	–	3
81–90	–	3
Total	72	63

is detected by growing them in an MTP, which is then detected quantitatively by spectrophotometer using an ELISA reader.

In this study, *E. coli* were recovered from urine samples, i.e. samples were inoculated on blood agar and incubated at 37°C for 24 hours resembling *in vivo* conditions. So, *E. coli* isolates forming biofilm *in vitro* will be able to synthesize biofilm *in vivo*.

Virulence factors like hemolysin,² hemagglutination,⁵ serum resistance,² gelatinase,² cell surface hydrophobicity,⁶ and siderophore production⁷ were detected and correlated with biofilm production in UPEC isolates.

Detection of virulence factors

- I. **Hemolysin:** The *E. coli* isolates were inoculated on 5% sheep blood agar and incubated over night at 37°C. The indicator of hemolysin production was the presence of a zone of complete lysis of erythrocytes around the colony and clearing of the medium.^{3,6,7}
- II. **Hemagglutination:** The test was carried out as per the direct bacterial hemagglutination test-slide method. One drop of RBC suspension was added to a drop of broth culture and the slide was rocked at room temperature for 5 minutes. Presence of clumping was taken as positive for hemagglutination. Mannose-sensitive hemagglutination was detected by the absence of hemagglutination in a parallel set of tests in which a drop of 2% W/V D-mannose was added to the red cells and a drop of broth culture. MRHA was detected by the presence of hemagglutination of 3% 'O' blood group human RBCs in the presence of 2% W/V D-mannose.⁸
- III. **Serum resistance:** Overnight culture of *E. coli* on blood agar plates were suspended in Hank's balanced salt solution (HBBS). Equal volume of this bacterial suspension and serum (0.05 ml) were incubated at 37°C for 3 hours. Then, 10 µl of this mixture was inoculated on blood agar plate and incubated at 37°C for 24 hours and viable count was determined. It is termed as sensitive when colony count drop to <1% of initial value.⁹
- IV. **Gelatinase test:** Gelatinase production was tested using gelatin agar. The plate was inoculated with test organism and incubated at 37°C for 24 hours. The plate was then flooded with 1% tannic acid solution. Development of opacity

around colonies was considered positive for gelatinase.¹⁰

- V. **Cell surface hydrophobicity:** This was done by salt aggregation test (SAT). One loopful of bacterial suspension in phosphate buffer was mixed with equal volume of ammonium sulfate solution of different molarity on a glass slide and rotated for 1 minute. *E. coli* strains with SAT value ≤ 1.25 M were considered cell surface hydrophobic.⁶

VI. Siderophore production assay: The test was done by using Chrome azurole sulfonate (CAS) agar diffusion assay. The CAS assay detected color change of CAS–iron complex from blue to orange after chelation of the bound iron by siderophores. A strong ligand was added to a highly colored iron dye complex, when the iron ligand complex was formed, the release of the free dye was accompanied by a color change.¹¹

The antibiotic susceptibility testing was performed by using standard antimicrobial agents (Hi Media, Mumbai) amoxycyclavulanic acid (30 µg), ceftizoxime (30 µg), cotrimoxazole (25 µg), gatifloxacin (5 µg), gentamicin (10 µg), nitrofurantoin (300 µg), norfloxacin (10 µg), as per CLSI.⁸ *E. coli* (ATCC 25922) was used as control strain.

Tests for ESBL detection were performed as per CLSI guidelines.⁸ Cefoxitin resistant and imipenem-resistant UPEC isolates were suspected to produce Amp C and MBL, respectively. These isolates were processed further for confirmation by Modified three-dimensional test⁹ and Modified Hodge test,⁸ respectively.

Statistical analysis used

The data were analyzed by using SPSS version 17.0. A two-sided *P*-value ≤ 0.05 was considered to be significant.

Results

Incidence of UTI was more common in females, i.e. 72 (53.3%) in comparison to males 63 (46.7%) and was more common in sexually active females of age group 21–30 years, 37 (27%) (Table 1). These cases were more common from gynecology/obstetrics 36 (27%) followed by surgery 34 (25%) and urosurgery 26 (19%).

The biofilm production was positive in 18 (13.5%) isolates, which were two times more common in female 12 (67%) as compared to males 6 (33%).

The presence of hemolysin, serum resistance, and gelatinase production was more in biofilm positive isolates, but no statistical significant value and correlation could be obtained (Table 2).

In the present study, there was prevalence of 100% MDR in biofilm-producing isolates out of which 88% were ESBL producers. The AmpC and MBL production was found in 22 and 6% isolates,

Table 2 Distribution of various virulence factors in biofilm positive and biofilm negative UPEC

Virulence factors	Biofilm producer % (N=18)	Non biofilm producer % (N=117)	P value
Hemolysin	44.4	48	≥0.05
Haemagglutination	66.7	77	≥0.05
Serum resistance	77.8	66	≥0.05
Hydrophobicity	50	62	≥0.05
siderophore production	88.9	88	≥.05
Gelatinase	66.7	57	≥.05

respectively. The coprevalence of ESBL and AmpC was found in 22% isolates (Table 3).

The incidence of resistance to antibiotics like amoxyclovanic acid, norfloxacin, cotrimoxazole, gatifloxacin, and gentamicin was more in biofilm producers as compared to non-biofilm producers. Nitrofurantoin was found to be 100% effective against these biofilm positive isolates. These biofilm-positive UPECs were found to be multidrug resistant, which was proved to be statistically significant ($P \leq 0.05$) (Fig. 1).

Discussion

Biofilm-associated infections have a major deleterious impact on artificial implants and it often serves as source of recurrent infections.¹⁰ These are reported to affect 90% of indwelling stents in patients.¹¹ In the urinary tract, bacterial biofilms develop on bladder

mucosa-producing persistent and recurrent UTIs, chronic cystitis, prostatitis, etc.¹² It has been reported that 10–15% of patients undergoing short-term catheterization develop UTI.

Biofilm-associated bacterial infections are difficult to eradicate using antibiotics. The matrix may also be involved in the protection of the bacteria against toxic molecules such as antimicrobials, hydroxyl radicals, and superoxide anions. The biofilm matrix could also inhibit wash out of enzymes, nutrients, or even signaling molecules that could then accumulate locally and create more favorable microenvironments within the biofilm.¹³

Research had documented that recurrent UTIs in women were commonly (74%) because of biofilm-producing strains.¹² Therefore, the study of factors contributing to biofilm formation is important to reveal the new therapeutics agents, which can be useful for the treatment of chronic and recurrent UTI.

In the present study, the incidence of biofilm in UPEC was 13.5%; many other studies have reported biofilm in UPEC isolates as 16 and 6%.^{14,15}

In the present study, it has been observed that there is greater co-prevalance (78%) of serum resistant and biofilm formation in UPEC isolates. Serum resistance is one of the properties, which make the microbe resist host immune defense mechanisms and resulting in long-term persistence of microorganisms in host tissue. Therefore, this property can be a contributor in biofilm formation by microbes.

In the same way, UPEC isolates that are able to hemagglutinate, produce gelatinase, and are hydrophobic in nature could survive in genitourinary tract, inspite of flush out action of ciliated epithelium of mucosal layer of tract. As hemagglutination is mediated by type 1 fimbriae, which help in attachment of bacteria to mucosal epithelium and initiate the first step of biofilm formation. It had been found that biofilm-producing *E. coli* strains showed a significantly greater type 1 fimbriae expression than non-biofilm-producing strains.¹²

Further aggravation of infection occurs by gelatinase production, which mediate the invasion of pathogenic microbe into the deeper tissue by clearing the connective tissue matrix. In this study, biofilm positive isolates were able to hemagglutinate and gelatinase production in 12 (68%) of UPEC isolates

Table 3 Prevalence of ESBL, AmpC, MBL and MDR in biofilm producers

	Biofilm producer % (n=18)
ESBL	88
AmpC	22
MBL	6
ESBL + AmpC	22
MDR	100

ESBL: Extended spectrum beta lactamases, MBL: Metallo beta lactamases, MDR Multi drug resistant.

Figure 1 Antibiotic resistance pattern of biofilm and non biofilm producers uropathogenic *E.coli* (n=135)

(Table 1). There was no difference in the production of siderophore in biofilm-positive and -negative isolates.

It has been found that biofilm made the micro-colonies impermeable to antibiotics and bound the agents at the outer surface of the matrix layer. The presence of other virulence factors in UPEC further complicate the situation, by protecting these isolates from action of antibiotics, and made the treatment of UTIs difficult by development of a resistance in clinical UTI isolate against antibiotics.

The prevalence of MDR in biofilm-producing UPEC has been reported¹⁰ more in comparison to biofilm negative isolates. Multiple drug resistance could be due to spread of hospital strains of UPEC, and the high prevalence of ESBL (88%), AmpC (22%) and MBL (6%) positive isolates is possibly due to the creation of selective drug pressure because of common use of cephalosporins and other antibiotics in our region.

In the present study, nitrofurantoin (100%) was effective against biofilm-positive UPEC isolates, irrespective of beta-lactamases production. Emergence of resistance in UPEC against fluoroquinolones, gentamicin, cotrimoxazole, and third generation cephalosporins have been documented in this study (Fig. 1). Similar facts were observed in other studies.^{10,13,14}

Increased prevalence of MDR in patients with UTI and emerging drug resistance to commonly used antibiotics due to production of beta-lactamase was of concern. Nitrofurantoin was the only drug, which was 100% effective in biofilm positive UPEC, and this drug can be a useful reserved drug for the treatment of UTI in catheterized and hospitalized patients. Knowledge of the nature of biofilm in UTI will help in deciding the more effective treatment guidelines for persistent and recurrent UTI.

Limitation of this study

No statistical significant relation could be obtained between different virulence factors and biofilm, which could be because of lower number of isolates and further molecular detection of beta-lactamases like ESBL, AmpC, and MBL could not be done because of the unavailability of the appropriate facilities in the department.

Disclaimer Statements

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definition of intellectual content, clinical studies, data acquisition and manuscript editing. MS: experimental studies and manuscript preparation.

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Conflicts of interest None.

Ethics approval This study was approved by the ethical committee of the institute.

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