BIOFILM-FORMING CAPABILITIES OF URINARY *ESCHERICHIA COLI* ISOLATES

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ABSTRACT

The aim of the present study was to investigate biofilm-forming capabilities of clinically isolated strains *Escherichia coli*, associated with different urinary tract infections. Biofilm production was detected in 36% of the isolates from UTI. Additionally some of virulence factors are estimated to find correlation between antibiotic resistance, hemolysins, morfotypes and biofilm production. Our data indicate that no combination of VFs was highly associated with biofilm production.

Keywords: antibiotic resistance, biofilm, UPEC, virulence factors

Introduction

Biofilms are defined as an assemblage of microbial cells that are irreversibly associated (not removed by gentle rinsing) with a surface and enclosed in a matrix of primarily polysaccharide material allowing growth and survival in sessile environment (4). Biofilms are typically characterized by dense, highly hydrated clusters of bacterial cells. These cells secrete extracellular polymeric substances that hold the cell aggregates together. In some cases, clusters of cells are separated by channels through which fluid can move. As the bacterial cells adapt to growth in these hydrated surface-associated communities, they express phenotypic traits that are often distinct from those that are expressed during planktonic growth. These phenotypic differences are manifested in various ways, depending on the species of bacteria. More than 50% of all bacterial infections reported involve biofilm formation (1). Biofilms formed by bacterial pathogens on medically relevant surfaces are difficult to eradicate and are thus often involved in the development of infections (2). Biofilm growth of pathogenic bacteria on tissues or indwelling devices often results in infections that have increased tolerance to antimicrobials and the host immune response. The mechanism of biofilm associated antimicrobial resistance seems to be multifactorial and may vary. Elucidating the physiology of biofilm-associated bacteria is necessary for understanding infections as well as other processes that are mediated by microorganisms (21).

Urinary tract infections (UTI) are one of the most common bacterial infections in humans. *Escherichia coli* has the ability to cause symptomatic UTI and asymptomatic bacteriuria (ABU) (5, 22). The ability of uropathogenic *E. coli* (UPEC) to cause symptomatic UTI is associated with the expression of a variety of virulence factors, including adhesins (e.g., type 1 and P fimbriae) and toxins (e.g., hemolysin) (8, 19).

Bacterial adherence not only contributes to colonization, but also to invasion, biofilm formation, and host cell damage. The two primary fimbrial adhesins associated with UPEC strains are type 1 and P fimbriae. Type 1 fimbriae are mainly associated with cystitis and confer binding to α-D-mannosylated proteins, such as uroplakins, which are abundant in the bladder. Expression of P fimbriae is primarily linked to pyelonephritic strains. P fimbriae recognize the α-D-galactopyranosyl-(1→4)-β-D-galactopyranoside moiety present in the globoseries of glycolipids located in the human kidney as well as on erythrocytes. Both type 1 and P fimbriae trigger host responses that include cytokine production, inflammation, and exfoliation of infected bladder epithelial cells (12, 19).
Growing antimicrobial resistance of bacterial pathogens has become an important problem worldwide. The development of antibiotic resistance in *E. coli* has important clinical implications. The development of resistance to older agents such as ampicillin and trimethoprim-sulfamethoxazole, as well as the emerging problem of fluoroquinolone resistance, may substantially limit choices of antibiotic for treatment (11).

The aim of this study was to investigate *in vitro* biofilm-forming capabilities of 50 clinically isolated strains *Escherichia coli*, associated with different urinary tract infections (pyelonephritis, cystitis or asymptomatic bacteriuria) and possible relations with hemagglutination patterns and other virulence factors (haemolysins, curli, fimbriae).

**Materials and Methods**

**Bacterial strains**

A total of 50 isolates of *E. coli* were collected at the Medical University – Plovdiv from patients having symptoms of different type UTIs. Strains were confirmed as *E.coli* with conventional methods and were cultured in appropriate broth (TSB, LB, Muller – Hinton broth, M63 broth).

**Screening of morphotype**

All strains were screened at least twice for colony morphology using previously described methods (17). In brief; colonies were inoculated onto LB agar without salts, containing 40 μg/mL Congo Red (Merck) and 20 μg/mL Coomassie brilliant blue (Sigma-Aldrich, St. Louis, MO) (CR agar). After inoculation, the CR agar plates were incubated at 37°C for 24 h or at 28°C for 72 h. All plates were visually examined and the morphotypes were categorised as: red, dry and rough (rdar) - indicating expression of curli fimbriae and cellulose, brown (bdar) – indicating expression of fimbriae but not cellulose, pink (pdar) – indicating expression of cellulose, but not fimbriae and smooth and white (saw) – indicating expression of neither cellulose nor fimbriae. M9 minimal medium was used to check for auxotrophy. Production of colanic acid was confirmed on plates with minimal glucose agar (MGA). Strains with milky shiny colonies were positive for colanic acid production.

**Biofilm assay**

The capacity to form biofilms was assayed in microtitre plates as described with modification (14). Briefly, cells were initially grown for 24 h in TSB at 37°C. Subsequently, 10 μl of overnight cultures were added to 100 μl M63 media in 96-well polystyrene microtitre plates (Nunc) and incubated for 24 h without shaking at 37 °C. To correlate biofilm formation with planktonic cell growth in each well, the planktonic cell fraction was transferred to new microtiter plates and the OD$_{600}$ was measured. Plates were washed three times with PBS and the biofilm was stained with 0.1% (w/v) crystal violet (CV) for 10 min. The excess crystal violet dye was removed and this was followed by washing the microtitre plates three times with PBS. To release the CV 75% ethanol was added to the wells and the absorbance was measured at 600 nm to estimate the amount of biofilm formed. The experiments were performed in duplicate.

**Agglutination assays and haemolysis.** The capacity of bacteria to express type 1 fimbriae was assayed by their ability to agglutinate yeast cells on glass slides (10). Mannose-resistant hemagglutination (MRHA) was assessed as previously described (3). Briefly, a 5% suspension (10 μl) of human type A red blood cells washed in phosphate-buffered saline (PBS) was mixed with a 10-μl bacterial suspension on glass slides in the presence and absence of D-mannose. The bacterial suspension was prepared by transferring cells from a freshly grown LB agar colony into 50 μl PBS. Hemolysin production was detected on sheep’s blood agar (7). Strains that produced a clear zone of lysis after incubation for 24 h at 37°C were considered positive.

**Antibiotic susceptibility testing**

Bacterial susceptibility to antimicrobial agents was determined by the disk diffusion method on Muller - Hinton agar (BB-NCIPD Ltd.) against twelve antibiotics which are often drugs of choice for treatment of UTIs. Antibiotic discs (Bioanalyse-Ankara) used are: ampicilline (AM), ampicillin/sulbactam (SAM), cefuroxime (CXM), cefotaxime (CTX), cefotaxime/clavulanic acid (CTC), nitrofurantoin (F), gentamycin (GN), amikacin (AK), nalidix acid (NA), ciprofloxacin (CIP), norfloxacin (NOR), trimethoprim/sulfamethoxazole (SXT). Detection of extended – spectrum β-lactamas (ESBLs) was done according to recommendations of Health Protection Agency for laboratory detection and reporting of bacteria with extended spectrum β-lactamas (13). Tests were performed by the double disc synergy test (DDST). *Escherichia coli* ATCC 25922 was used as control strain for susceptibility studies.
Results and Discussion

Escherichia coli strains are the most frequent cause of urinary tract infections. Biofilm formation allows the strains to persist a long time in the genitourinary tract and interfere with bacterial eradication. We investigated the possible relationships between in vitro biofilm formation and the presence of some urovirulence factors and antibiotic resistance. A total of 50 E. coli strains collected from patients with cystitis (15 strains), pyelonephritis (25 strains), prostatitis (5 strains) and ABU (5 strains) were analyzed for in vitro biofilm formation. In current study 12 (24%) strains were in vitro positive for biofilm production (Fig. 1).

![Fig. 1. Biofilm-forming abilities of some of urinary Escherichia coli isolates](image1)

This is relatively lower than biofilm forming capabilities of UPEC strains reported in other studies (16, 20). These authors reports prevalence of biofilm production about 43% for strains isolated from patients with cystitis, 40% for pyelonephritis and 42% for bacteraemic E. coli strains (20) and even higher abilities among the E.coli strains (53%) isolates from patients with bacteraemia of urinary tract (16). Soto et al. (2007) reported a high, 63%, prevalence of biofilm formation among strains from patients with prostatitis. These results suggest that UPEC employ there biofilm-forming abilities to invade and sucessfully occupy tissues in urogenital tract. We didn’t established strong association between biofilm forming abilities and type of UTI with which strains were associated.

Haemagglutination and haemolysis were employed to investigate presence of type 1 fimbria and hemolysins. We found that 12 (24%) of E.coli strains produce α hemolysin. There is no correlation between biofilm formation and hemolysin production. Type 1 fimbriae, which promote adhesion to host epithelial cells, have been found to be important in the initial steps of biofilm formation (15). We established relatively low presence (16%) of type 1 fimbriae among investigated strains while 23 (46%) display MRHA activity, which can be mediated by P fimbriae, X, FIC and DR fimbriae (6). One of the reasons may be an adaptation after successful colonization and transition to commensalism with loss of some virulence factors.

Curli fimbriae and/or cellulose are expressed by E. coli, Salmonella spp. and other Enterobacteriaceae (18, 23). The role of curli and E. coli exopolysaccharide colanic acid was determined. Bacteria expressing curli fimbriae and cellulose display a red, dry and rough colony morphology (rdar) on Congo red agar plates. Disruption of any of these components leads to the development of distinct colony morphology types. Deficiency in fimbriae and cellulose synthesis causes a smooth and white (saw) colony appearance, a defect in cellulose synthesis leads to brown (bdar) colonies and a defect in curli fimbriae expression to a pink (pdar) morphotype (18). None of the investigated strains was with bdar morphotype, 22 (44%) were rdar positive, 18 (36%) were pdar positive and 10 (20%) were with saw morphotype (Fig. 2).

![Fig. 2. Different morfotypes among investigated strains rdar - expression of curli fimbriae and cellulose; pdar - expression of cellulose, but not fimbriae; saw - expression of neither cellulose nor fimbriae](image2)

Only 7 strains (14%) express colanic acid and there had no participation in biofilm-formation. We found that morphtype of strains varied between different type UTI. None of these morphtypes are prevalent among investigated strains. Other authors report prevalence of the rdar morphtype since this type has been associated with virulence.

We investigated antibiotic resistance patterns of strains against 12 drugs currently used in therapy of UTI (Table. 1). The investigated strains displayed relatively high resistance...
against tested antibiotics. Additionally 19 (38%) strains are
with multidrug resistance phenotype and 8 (16%) strains
produce ESBLs. Our results are with agreement with other
reports for growing resistance of UPEC. At the presence we
didn’t find a relation between higher resistance and biofilm
forming capabilities of isolates.

Conclusions
Biofilms were detected in vitro from 24% of investigated
E.coli strains, which showed different morphotypes resulted
from production of curli fimbriae, cellulose and colanic acid.
E.coli was the most frequent isolate from urinary tract
infections and our data show that strains had vastly antibi
resistance to conventional antibiotics. We suppose that
biofilm formation from these strains is complex work of
many factors at all stages of process. It seems that each strain
specifies production and expression of multiple types of cell
surface structures, which are influenced by environmental
conditions. Among the strains investigated in our study
biofilm production was not significantly associated with any
virulence determinant or combination of virulence
determinants.

TABLE 1
Antibiotic resistance of investigated 50 urinary Escherichia
coli isolates

<table>
<thead>
<tr>
<th>Antimicrobial agent *</th>
<th>Resistance isolates n (%)</th>
</tr>
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<tbody>
<tr>
<td>AM</td>
<td>37 (74)</td>
</tr>
<tr>
<td>SAM</td>
<td>25(50)</td>
</tr>
<tr>
<td>CXM</td>
<td>15 (30)</td>
</tr>
<tr>
<td>CTC</td>
<td>14 (28)</td>
</tr>
<tr>
<td>CTC</td>
<td>4 (8)</td>
</tr>
<tr>
<td>F</td>
<td>1 ( 2)</td>
</tr>
<tr>
<td>GN</td>
<td>20 (40)</td>
</tr>
<tr>
<td>AK</td>
<td>6 (12)</td>
</tr>
<tr>
<td>NA</td>
<td>29 (58)</td>
</tr>
<tr>
<td>CIP</td>
<td>19 (38)</td>
</tr>
<tr>
<td>NOR</td>
<td>23 (46)</td>
</tr>
<tr>
<td>SXT</td>
<td>36 (72)</td>
</tr>
</tbody>
</table>

* - AMP – Ampicillin; SAM – Ampicillin/sulbactam; CXM – Cefuroxime;
CTX – Cefotaxime; CTC – Cefotaxime/Clavulanic acid; F – Nitrofurantoin;
GN – Gentamycin; AK – Amikacin; NA - Nalidix acid; CIP –
Ciproflaxacin; NOR – Norfloxacin; SXT – Trimethoprim/sulfametoxazole.

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