Research Article

# Biofilm Formation in Nonmultidrug-resistant *Escherichia coli* Isolated from Patients with Urinary Tract Infection in Isfahan, Iran

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## Abstract

**Background:** *Escherichia coli* is a Gram-negative, opportunistic human pathogen in which increasing antibiotic resistance is a great concern for continued human survival. Although biofilm formation is a mechanism that helps *E. coli* to survive in unfavorable conditions, according to the importance of biofilm formation in developing the antibiotic resistance here, we studied the relation between antibiotic resistance and *in vitro* qualitative rating method biofilm formation in *E. coli* isolated from patients with urinary tract infection (UTI). **Materials and Methods:** The clinical isolates of *E. coli* ($n$ = 100) were collected from urine of patients with UTI attending Isfahan Alzahra hospital. The strains were confirmed as *E. coli* using biochemical tests and molecular method. The Kirby-Bauer disk diffusion tests were done according to the Clinical and Laboratory Standards Institute protocol, and the biofilm synthesis was performed by microplate method. The binary logistic test was applied and $P < 0.05$ was considered statistically significant. **Results:** Our results showed a high outbreak of multidrug-resistant (MDR) *E. coli* strains (73%) and the highest resistance was observed toward ampicillin. The prevalence of biofilm producer isolates was 80% that 29% produced strong biofilm. The distribution of non-MDR isolates was high among strong biofilm producers, which shows a significant negative correlation between biofilm production and MDR pattern ($P < 0.001$). **Conclusions:** We found a negative correlation between MDR phenotype and biofilm formation capacity. This transmits the concept that more antibiotic susceptibility of strong biofilm producers may be due to the reduced exposure to multiple antibiotics.

**Keywords:** *Antibiotic resistance, biofilm formation, Escherichia coli, urinary tract infections*

## Introduction

Urinary tract infections (UTIs) are probably the most widely recognized bacterial diseases, influencing 150 million individuals every year around the world. UTIs are the considerable reason of morbidity in females of any age, infant boys, and older men.[1] *Escherichia coli* represents 80%–90% of causative uropathogens which is responsible for complicated and uncomplicated UTIs.[2] Multidrug-resistant (MDR) UTIs are turning out to be progressively hard to treat because of the variety of antibiotic- resistance mechanisms. Of particular worries are members of the *Enterobacteriaceae* family including *E. coli* which is capable of acquiring plasmids encoding extended-spectrum β-lactamases.[3] These plasmids rapidly induce resistance to the third generation of cephalosporins and also to other antibiotics.[4-6] In addition, this is not the only cause of antibiotic treatment failure, and for many situations, it may not be the main factor.[7] In fact, biofilm formation seems to be an important consideration for pathogenesis and the reason of therapeutic failure, especially in some of the device-associated infections such as long-term catheterized patients with urinary tract infections.[8] Biofilms consider as assemblages of microorganisms attached to a surface. It has become obvious that sessile bacterial cells in the biofilms express properties distinct from planktonic cells, for example, the higher resistance to antibiotics and antibacterial agents which leads to survival in hostile environments.[9] Other researches in Iran also show a high tendency of *E. coli* to produce biofilm. Tajbakhsh *et al*. and Karimi *et al*. showed that 80% and 68% of *E. coli* isolates were capable to produce biofilm respectively.[10,11] Because of the significance of biofilm production in pathogenesis of *E. coli* and antibiotic resistance, the correlation between antibiotic resistance and biofilm formation was determined in the present study.



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## Materials and Methods

### Bacterial strain collection and identification

A total of 100 clinical isolates of *E. coli* were obtained from Isfahan Alzahra Hospital during March 2015 to September 2015. All of the isolates were collected from urine samples during 6 months, and they were identified by phenotypic and biochemical methods including Gram-staining, glucose, and lactose fermentation (Triple-sugar iron agar medium), $H_2S$ production, motility, indole, methyl red, Voges-Proskauer, Simmons citrate, and phenylalanine deaminase tests.

### Genotypic confirmation of *Escherichia coli* by polymerase chain reaction method

The polymerase chain reaction (PCR) was done for the verification of *E. coli* strains by targeting the *uidA* gene for β-glucuronidase. PCR amplification was realized in a final volume of 20 μL containing 0.5 μL of each primer (forward primer: 5'-ATCACCGTGGTGACGCATGTCGC-3' and reverse primer: 5'-CACCACGATGCCATGTTCATCTGC-3'), 10 μL of PCR master mix (Ampliqon red, Denmark), 8.5 μL of RNase-free water, and 0.5 μL (500 ng) of a DNA extract denaturation at 94°C, followed by 30 cycles of 94°C for 1 min, 1 min of annealing at 50°C, and 1 min of extension at 72°C followed by a final extension step of 7 min at 72°C. The product size was 486 base pair and *E. coli* ATCC 25922 was used as positive control.[12]

### Antimicrobial susceptibility testing

All of the 100 strains were tested for antibiotic susceptibility by disk diffusion method according to the Clinical and Laboratory Standards Institute 2014 guidelines with commercially available disks (MAST, Merseyside, UK). The following antibiotic disks were used: ciprofloxacin (5 μg), amikacin (30 μg), cefotaxime (30 μg), gentamicin (10 μg), nitrofurantoin (300 μg), trimethoprim/sulfamethoxazole (1.75/23.75 μg), cefoxitin (30 μg), imipenem (10 μg), cefepime (30 μg), ceftazidime (30 μg), cefazolin (30 μg), tetracycline (30 μg), piperacillin-tazobactam (100/10 μg), and ampicillin (10 μg). Antimicrobial susceptibility of the strains was analysis with the WHONET software.

### Biofilm formation assay

Biofilm production assays were performed following a previously explained protocol.[13,14] The isolates were incubated overnight in 5 ml LB medium at 35.5°C. A volume of 1.3 μL from overnight cultures (c. $8.7 \times 10^5$ CFU) was added to 130 μL of M9 broth media in wells of polyvinyl chloride 96-well microtiter plates and incubated without shaking at 30°C for overnight. Each bacterial suspension was inoculated in three wells of the microtiter plate. Growth optical densities (OD) were measured at λ = 620 nm by multiplate reader (Biotek, USA). Then, wells were washed once with 150 μL sterile saline. The wells were dried for 20 min and stained with 130 μL 1% crystal violet for 5 min. Then, the colorant was discarded and the stained biofilms were washed gently with 180 μL of distilled water (four times) and air-dried for 1 h. The absorbed dye was solubilized in 130 μL of absolute ethanol and ODs were read at 540 nm. The extent of biofilm formation was calculated using the formula: SBF = (AB − CW)/G, where SBF is the specific biofilm formation index, AB is the $OD_{540}$ nm of the stained bacteria, CW is the $OD_{540}$ nm of the stained control wells containing absolute medium without bacteria, and G is the $OD_{620}$ nm of cell growth in media. *E. coli* ATCC 25922 was used as positive control and the culture medium used as negative controls. The isolates were categorized as follows: SBF≥1.10: strong biofilm formation, SBF = 0.70–1.09: moderate biofilm formation, SBF = 0.35–0.69: weak biofilm formation, and SBF <0.35: negative biofilm formation.

### Statistical analyses

The binary logistic regression analysis and the SPSS version 23 software were used to study the correlation between the SBF and antibiotic resistance.

## Results

All isolates were confirmed as *E. coli* by biochemical tests and PCR method [Figure 1] and were examined by further tests.

### Antimicrobial susceptibility testing

Our findings were analyzed using WHONET, version 5.6. All susceptibility data are summarized in Table 1. Resistance to ampicillin was the most common (71%), and 73% of the isolates were not susceptible to at least one agent in three or more antimicrobial categories which defined as MDR[15] and 27% of the isolates were considered

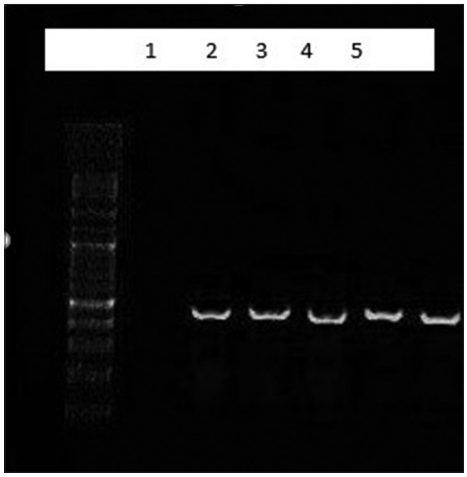


**Figure 1: Electrophoresis of polymerase chain reaction product of *uidA* gene for β-glucuronidase on the agarose gel 1%. L: DNA ladder 100 bp, 1: Negative control, 2: Positive control, 3, 4, 5: Positive bands for bacterial samples**

as non-MDR. The minimum resistance was related to imipenem.

### Biofilm formation analysis

Microplate method showed that 80% of the isolates tend to form biofilm and 20% not producing any biofilm. The details are summarized in Table 2.

### Correlation between biofilm formation and multidrug-resistant strains

The simple logistic regression test analysis indicated that there is a significantly negative correlation between biofilm formation capacity and the inherent ability of bacteria to show multidrug resistance ($P < 0.001$). Our findings indicated that non-MDR isolates tended to form more robust biofilm formation. Among 29 strong biofilm producers, 69.2% (n = 18) were non-MDR isolates and 38% (n = 11) were MDR isolates. All of the negative and weak biofilm producers were MDR isolates [Table 3]. These ratios revealed that the population that represented more robust biofilm synthesis likely contained higher population of non-MDR isolates [Figure 2].

## Discussion

Biofilm-forming bacteria develop chronic infections since they indicate increased tolerance to antibiotics.[16] The correlation between biofilm synthesis and antibiotic resistance is of notable concern to biomedical studies. However, it is still doubtful whether there is any quantitative relationship between biofilm formation and antibiotic resistance. Over the recent decades, different researches have yielded incompatible results.[17] Our results not only provide information about the balance between biofilm formation and antibiotic resistance of *E. coli* isolated from patients with UTI that can help this organism to improve its viability but also can serve as a guidance for the antibiotic therapy of biofilm infections among UTI patients. We found that the prevalence of MDR isolates was about 73%, which is close to the reports of recent studies in Iran,[18,19] and we demonstrated a high tendency for biofilm formation among the clinical isolates of *E. coli*. Biofilm synthesis in *E. coli* promotes the persistence in device-related infections.[20] Here, we concluded that non-MDR isolates tend to produce stronger biofilms. It seems that biofilm formation is a mechanism for a better survival of bacteria, particularly when resistance level is not sufficiently high. However, previous studies have not reported any clear quantitative correlation between antibiotic resistance and biofilm formation.[17] Atashili *et al*. did not find a significant correlation in biofilm production among MDR and non-MDR isolates of *Staphylococcus aureus*.[21] Gurung *et al*. studied 60 isolates of *Acinetobacter baumannii* and reported a positive relationship between antibiotic resistance and biofilm formation.[22] However, Qiu *et al*. evaluated biofilm-forming capacity among 272 *A. baumannii* isolates in the absence of antibiotic-mediated stress and indicated that antibiotic-susceptible isolates produced stronger biofilms.[17] Perez *et al*. displayed an inverse relationship between meropenem resistance and biofilm formation in 116 *A. baumannii* isolates.[23] The important thing to note is that the Kirby-Bauer disk diffusion test demonstrates the original pattern of bacterial antibiotic resistance and the best proposed method for realizing the biofilm-specific resistance is minimum biofilm eradication

**Table 1: The susceptibility test among *Escherichia coli* isolated from patients with urinary tract infections**

| Antibiotics | Percentage isolates | | |
|---|---|---|---|
| | Susceptible | Intermediate | Resistant |
| Amikacin | 56 | 33 | 11 |
| Ampicillin | 29 | 20 | 51 |
| Cefazolin | 73 | 0 | 27 |
| Cefepime | 61 | 29 | 10 |
| Cefotaxime | 34 | 26 | 40 |
| Cefoxitin | 79 | 12 | 9 |
| Ceftazidime | 37 | 29 | 34 |
| Ciprofloxacin | 56 | 9 | 35 |
| Gentamicin | 67 | 13 | 20 |
| Imipenem | 98 | 2 | 0 |
| Nitrofurantoin | 47 | 33 | 20 |
| Piperacillin/tazobactam | 59 | 23 | 18 |
| Tetracycline | 42 | 0 | 58 |
| Trimethoprim/sulfamethoxazole | 43 | 1 | 56 |

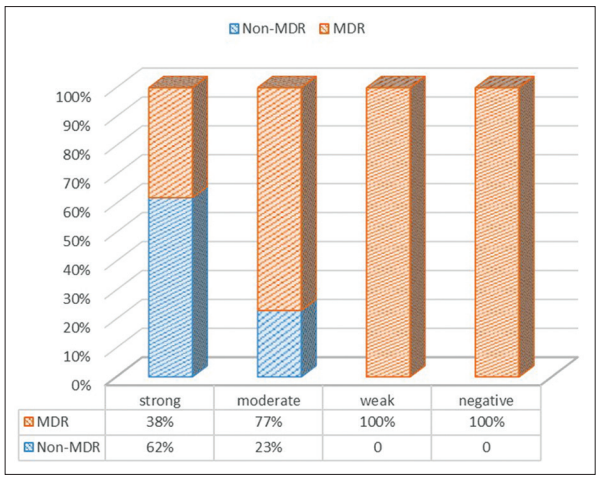


**Figure 2: Distribution of multidrug-resistant and nonmultidrug-resistant isolates among various biofilm formation capacities represented as a percentage stacked bar graph. Stronger biofilm producer population contained a larger proportion of nonmultidrug-resistant isolates**

**Table 2: Results of biofilm formation by microplate method**

| Strength of biofilm formation | No producer | Weak producer | Moderate producer | Strong producer | Total |
|---|---|---|---|---|---|
| *n* (%) | 20 | 17 | 34 | 29 | 100 |

**Table 3: Biofilm-forming capacities of *Escherichia coli* with different antibiotic-resistant phenotypes**

| Biofilm-forming capacities | Values | MDR | | Total |
|---|---|---|---|---|
| | | Non-MDR | MDR | |
| Biofilm negative | Count | 0 | 20 | 20 |
| | Percentage within biofilm | 0.0 | 100.0 | 100.0 |
| Weak | Count | 0 | 17 | 17 |
| | Percentage within biofilm | 0.0 | 100.0 | 100.0 |
| Moderate | Count | 9 | 25 | 34 |
| | Percentage within biofilm | 23 | 77 | 100.0 |
| Strong | Count | 18 | 11 | 29 |
| | Percentage within biofilm | 62 | 38 | 100.0 |
| Total | Count | 27 | 73 | 100 |
| | Percentage within biofilm | 27 | 73 | 100.0 |

MDR: Multidrug-resistant

concentration (MBEC)[22] in which the strains are allowed to produce biofilm and then treated with antibiotics so that the increased antibiotic resistance in biofilm which is due to the failure of the antimicrobial to penetrate the biofilm and activation of quorum-sensing genes and multidrug efflux pumps can be observed.[23] Alves *et al*. determined that half of biofilm-producing bacteria in clinical isolates from urinary tract infections were MDR profile, and among resistant isolates to each antibiotic, the number of negative biofilm producers were more than biofilm producers except about fosfomycin, nitrofurantoin, imipenem, and also, among 22 isolates of biofilm positive *E. coli*, only 6 isolates were MDR and 16 isolates were non-MDR.[24] The important point is that the Kirby-Bauer disk diffusion test demonstrates the original pattern of bacterial antibiotic resistance while the best proposed method for realizing the biofilm-specific resistance is MBEC[25] in which the strains are allowed to produce biofilm and then treated with antibiotics so that the increased antibiotic resistance in biofilm which is due to the failure of the antimicrobial to penetrate the biofilm and activation of quorum-sensing genes and multidrug efflux pumps can be observed.[26] Ordinary culture methods (e.g., Kirby-Bauer disk diffusion test) display only the properties of planktonically growing bacteria, therefore, present misleading results that do not reveal the developed resistance of the bacteria living in biofilms. Methods to examine biofilm-growing bacteria have already been developed, but their clinical relevance with attention to prediction of clinically successful therapy awaits confirmation.[16]

## Conclusions

The results from our study indicated that there is a negative correlation between antibiotic-resistant phenotype and biofilm formation capacity. This implies that biofilm formation is a mechanism that helps bacteria to get better survival, particularly in isolates with resistance level not sufficiently high and more antibiotic susceptibility of strong biofilm producers may be due to the reduced exposure to multiple antibiotics.

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### Conflicts of interest

There are no conflicts of interest.

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