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The Prevalence of *acrA* and *acrB* Genes Among Multiple-Drug Resistant Uropathogenic *Escherichia coli* Isolated From Patients With UTI in Milad Hospital, Tehran

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Abstract

Background: Urinary tract infection (UTI) is one of the most common infectious diseases and nosocomial infections worldwide, and uropathogenic *Escherichia coli* is the primary cause of UTI. Due to increased antibiotic resistance and the emergence of multidrug resistant (MDR) UPEC clones, the treatment of UTI is difficult. The occurrence of MDR in *E. coli* has been attributed to the AcrAB-TolC complex of efflux pumps.

Objectives: The aim of this study was to complete a frequency evaluation of *acrA* and *acrB* genes among UPEC MDR strains isolated from patients with UTI who were admitted to Milad hospital in Tehran.

Methods: For 123 UPEC strains that were isolated and diagnosed from the urine samples of patients using biochemical tests, antibiotic susceptibility was carried out using the disc diffusion method according to CLSI guidelines. Isolates that were resistant to at least one antimicrobial agent in three or more of the categories were considered to be MDR. The presence and frequency of *acrA* and *acrB* genes was determined using PCR.

Results: The rates of antibiotic resistance to ampicillin, cefalotin, tetracycline, cefazolin, ceftriaxone, ceftizoxime, ceftazidime, ciprofloxacin, and cotrimoxazole were 82.9%, 78.1%, 61.1%, 49.5%, 38.2%, 30.2%, 26.1%, 42.2%, and 60.1%, respectively. The isolates were most sensitive to nitrofurantoin (95.9%), gentamicin (77.2%), and amikacin (71.5%). A total of 78% of the isolates were MDR. The frequency of the *acrA* gene was 82.90%, the *acrB* gene was 95.90% and *acrA* + *acrB* was 95.90%. There was no significant difference between *acrA* and *acrB* frequency relating to bacterial antibiotic resistance.

Conclusions: Our results showed that ways to control the treatment of UTI for the prevention of MDR occurrence should be sought. For a better study of efflux pumps, a comprehensive and detailed study regarding the presence of efflux pumps gees is required.

Keywords: Uropathogenic *Escherichia coli*, Multi-Drug Resistant, *acrA* and *acrB*

1. Background

Urinary tract infections (UTIs) are one of the most common types of infections, second only to respiratory tract infections; every year, about 150 million people worldwide are infected with UTI (1).

Of the many Gram-negative bacteria that may cause UTI, *Escherichia coli* is the most common (2). *E. coli* is a commensal organism that is found in the human gut; however, it can also be pathogenic and often causes UTIs.

The appropriate treatment for UTIs is the selection of effective, accessible, high-performance antibiotics, but in the past decade, the number of antibiotics to which bacteria have developed resistance has increased considerably (3-5). As a consequence, some agents are no longer useful for the treatment of infections, and bacteria that are resistant to antimicrobial agents, including bacteria with multidrug resistance (MDR), are an increasing problem in healthcare in both community and hospital settings (6, 7).

There are a variety of antibiotic resistance mechanisms in Gram-negative and Gram-positive bacteria. The intrinsic resistance of Gram-negative bacteria to certain antibiotics relative to Gram-positive bacteria is a result of the activity of efflux systems (8). Efflux pumps are transport proteins involved in the extrusion of toxic substrates (including virtually all classes of clinically relevant antibiotics) from within cells into the external environment that are found in both Gram-negative and Gram-positive bacteria and in eukaryotic organisms (9). Pumps that transport several compounds can be associated with MDR. There are five major families of efflux transporters: major facilitator (MF), multidrug and toxic efflux (MATE), resistance-nodulation-division (RND), small multidrug resistance (SMR), and ATP-binding cassette (ABC) (10, 11). The AcrAB-TolC efflux pump, from the RND family, is the most important efflux system in *E. coli*, *Klebsiella*, and *Salmonella typhimorium* (12). These efflux pumps comprise a transporter (efflux) protein (for

example, *AcrB* that is encoded by the *acrB* gene), which is located in the inner (cytoplasmic) membrane of a bacterium; an accessory protein (also known as a membrane-fusion protein, such as *AcrA* that is encoded by *acrA* gene), which is located in the periplasmic space; and an outer-membrane protein (also known as an outer membrane protein channel, such as TolC that is encoded by the *tolC* gene), which is located in the outer membrane of a bacterium (13).

2. Objectives

Due to the role of *E. coli* in UTIs, the increasing incidence of MDR, and the role of efflux pumps in MDR, the aim of this study was to determine the MDR of *E. coli* strains isolated from UTI patients in Tehran Milad hospital and frequency of the efflux pump genes among those isolates.

3. Methods

This descriptive study was performed on 123 *E. coli* strains isolated from 200 patients with UTIs who were admitted to the clinical laboratory of Milad hospital in Tehran. Midstream urine sample of patients were collected in sterile bottles. The samples with significant bacteriuria (more than 10^5 cfu per mL) were selected for this study.

3.1. Cultivation and Bacterial Isolation

The samples were directly inoculated on MacConkey agar and EMB agar plates after overnight incubation at 37°C. The biochemical identification was carried out using bacterial culturing on TSI agar, a SIM medium, Simmons' citrate agar, and an MRVP broth.

3.2. Antibiotic Susceptibility Testing

Antibiotic susceptibility was performed on Muller-Hinton agar (Merck, Germany) using the Kirby Bauer disc diffusion method according to the 2014 CLSI guidelines. The antibiotic discs (padtanteb) and concentrations (μg) that were used were as follows: ciprofloxacin (CP; 5 μg), cotrimoxazole, (SXT; 25 μg), gentamicin (CN; 10 μg), amikacin (AK; 30 μg), nitrofurantoin (FM; 20 μg), ceftazidime (CAZ; 30 μg), ampicillin (Am; 10 μg), ceftriaxone (CRO; 30 μg), cefazolin (CZ; 30 μg) ceftizoxime (CT; 30 μg), cefalotin (CF; 30 μg), and tetracycline (TE, 30). The results of the testing were interpreted as either susceptible or resistant according to criteria recommended by the CLSI and the manufacture protocols. The isolates that were resistant to at least one antimicrobial agent in three or more of the categories were considered MDR. Quality control was tested by *E. coli* ATCC25922.

3.3. DNA Extraction and Molecular Identification

The DNA of all isolates was extracted using a genomic DNA isolation kit (Gene Transfer Pioneers, Iran). Before DNA extraction *E. coli* strains were cultured in an LB broth at 37°C for 18 hours. Bacterial confirmation was achieved using the PCR technique on the *E. coli16SrRNA* gene. The primers that were used in this study are listed in Table 1.

The concentration and amount of materials used for the amplification of *acrA* and *acrB* genes by PCR are listed in Table 2. Amplification was performed in 30 cycles: 1 minute of at 94°C, 1 minute of annealing at 52°C, 1 minute at 72°C, and a final extension at 72°C for 5 minutes. The PCR products were electrophoresed by a gel agarose (Sinaclon, Iran) and visualized by a UV transilluminator (Ultraviolet Transilluminator, UVT-20M, KIGEN). The positive control for the *acrA* and *acrB* genes was *E. coli* ATCC25922.

3.4. Statistical Analysis

Statistical analysis was done using the statistical package of SPSS Windows version 16. A χ^2 test was used to study the correlation between the prevalence of the *acrA* and *acrB* genes and the type of antibiotic resistance of UPEC isolates. A value of $P < 0.05$ was considered statistically significant.

4. Results

From 2185 patient urine samples, 200 (9.1%) UTIs were diagnosed and 123 (61.5%) uropathogenic *E. coli* strains were isolated from 200 samples of UTI based on the results of biochemical tests.

The antibiogram test results of all 123 strains showed that the highest resistance to antibiotics belonged to ampicillin (82.9%), cefalotin (78.1%), and tetracycline (61.1%). Antibiotic resistance to cefazolin (49.5%), ceftriaxone (38.2%), ceftizoxime (30.2%), ceftazidime (26.1%), ciprofloxacin (42.2%), and cotrimoxazole (60.1%) was also reported (Figure 1). The highest sensitivities belonged to nitrofurantoin (95.9%), gentamicin (77.2%), and amikacin (71.5%) (Table 3). The evaluation of the antibiotic resistance of UPEC strains to more than two classes of antibiotics showed that 27.1% of strains were resistant to three classes, 17% were resistant to four and five classes, 16.2% were resistant to six classes, and 6.5% were resistant to seven classes (Figure 2). Therefore, the frequency of MDR strains was 96 (78%).

The electrophoresis analysis of the PCR products of the *acrA* and *acrB* genes showed bands with a size of 107 bp for the *acrA* and *acrB* genes. The band size of the *16S rRNA* gene was 723 bp (Figure 3).

The frequency of *acrA* and *acrB* genes among UPEC isolates were 102 (82.9%) and 118 (95.9%), respectively. A total of 102 (82.9%) isolates had both genes (Figure 4).

Table 1. The Nucleotide Sequences of Primers

Genes	Nucleotide Sequences of Primers	Size, bp
16SrRNA	5' -CGA GTG GCG GAC GGG TGA GT-3'	723
	5' -TCG ACA TCG TTT ACG GCG TGG A-3'	
acrA	5' -CTCTCAGGCAGCTTAGCCCTAA -3'	107
	5' -TGCAGAGGTTCAGTTTTGACTGTT-3'	
acrB	5' -GGTCGATTCCGTTCTCCGTTA-3'	107
	5' -CTACCTGGAAGTAAACGTCATTGGT-3'	

Table 2. Concentration and Amount of Materials Used for the Amplification of *acrA* and *acrB* Genes by PCR

Materials	Amount
H_2O	9.7 μL
10X PCR buffer	1.5x
$MgCl_2$	2 mM
dNTP	0.2 Mm
AcrA or AcrB-F	4 pmol
AcrA or AcrB-R	4 pmol
16S rRNA(F)	4 pmol
16S rRNA(R)	4 pmol
DNA	100 ng
Taq DNA polymerase	1.5 Unitt
Total volume	15 μL

Table 3. Antimicrobial Susceptibility Patterns of Uropathogenic *E. coli* Isolates[a]

Antibiotics	Codes	Discs	Resistance	Intermediate	Sensitive
Ampicillin	AM	10 μg	102 (82.9)	5 (4.1)	16 (13)
Cefalotin	CF	30 μg	96 (78.1)	6 (4.8)	21 (17.1)
Cefazolin	CZ	30 ug	61 (49.5)	48 (39.2)	14 (11.3)
Ceftriaxone	CRO	30 ug	47 (38.2)	5 (4.1)	71 (57.7)
Ceftizoxime	CT	30 ug	37 (30.2)	10 (8.1)	76 (61.7)
Ceftazidime	CAZ	30 ug	32 (26.1)	14 (11.3)	77 (62.6)
Tetracycline	TE	30 ug	75 (61.1)	10 (8.1)	38 (30.8)
Nitrofurantoin	FM	300 ug	2 (1.7)	3 (2.4)	118 (95.9)
Amikacin	AN	30 ug	5 (4.1)	30 (24.4)	88 (71.5)
Gentamicin	GM	10 ug	16 (13.1)	12 (9.7)	95 (77.2)
Ciprofloxacin	CP	5 ug	52 (42.2)	7 (5.7)	64 (52.1)
Cotrimoxazole	SXT	25ug	74 (60.1)	19 (15.5)	30 (24.4)

[a]Values are expressed as No. (%).

The prevalence study of the *acrA* and *acrB* genes among isolates based on antibiotic resistance showed that the highest incidence (100%) of isolates with both genes was among isolates with amikacin or nitrofurantoin resistance. However, only two isolates were resistant to amikacin, and only five were resistant to nitrofurantoin, which was not significant ($P > 0.05$). The lowest frequency of *acrA* was 81.2% in gentamicin-resistant isolates, and the lowest frequencies of *acrB* was 93.6% ceftriaxone-resistant isolates and 93.7% for gentamicin-resistant isolates (Table 4). There was no significant relationship between the frequency of *acrA* and *acrB* genes and antibiotic resistance ($P > 0.05$).

5. Discussion

Improving UTIs caused by UPEC strains often requires antibiotic treatment. However, long-term antibiotic resistance of the bacteria involved in UTIs to antibiotics makes the disease more severe and more likely to recur.

Table 4. The Frequency of the *acrA* and *acrB* Genes Among Antibiotic-Resistant Bacteria[a]

Gene	AM	CT	SXT	GM	CP	AN	TE	FM	CRO	CF	CAZ	CZ
acrA	83.3	86.4	85.1	81.2	88.4	100	85.3	100	87.2	84.3	87.5	90.1
acrB	96	94.5	94.5	93.7	96.1	100	97.3	100	93.6	95.8	93.7	96.7

[a] Values are expressed as %.

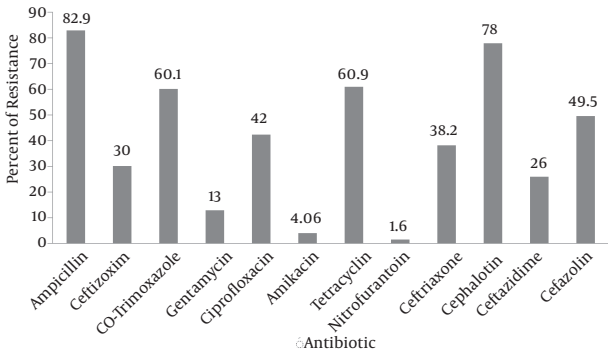

Figure 1. The Frequency of Antibiotic Resistance of UPEC Isolates

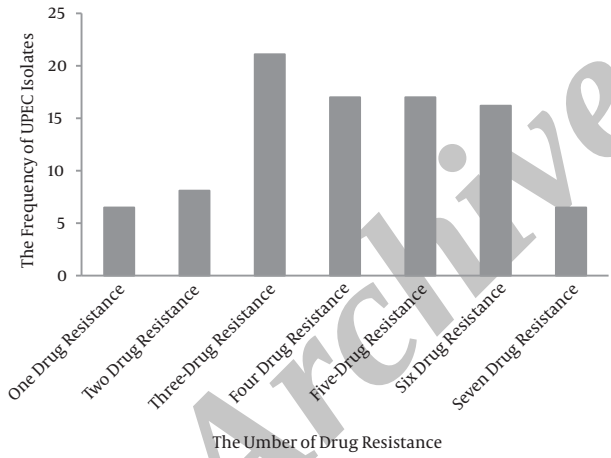

Figure 2. The Frequency of UPEC Isolates That Were Resistant to a Number of Antibiotic Classes

Antibiotic resistance in uropathogenic *E. coli* is rising today, which has led to costly problems for the health sectors of different countries. In this study, the antibiotic susceptibility of UPEC strains isolated from patients with UTI who were referred to Milad hospital in Tehran was determined. The results showed that the isolates were most resistant to ampicillin (82.9%), cefalotin (78.1%), and tetracycline (61.1%). The isolates were most sensitive to nitrofurantoin (95.9%), gentamicin (77.2%), and amikacin (71.5%). Antibiotic resistance to cefazolin (49.5%), ceftriaxone (38.2%), ceftizoxime (30.2%), ceftazidime (26.1%), ciprofloxacin (42.2%) and cotrimoxazole (60.1%) was also reported.

In a study by Shariff et al., the UPEC isolates showed the highest sensitivity carbapenems (100%), cefoperazone/sulbactam (95.6%), piperacillin/tazobactam 92.2%, and ampicillin/sulbactam 62.3%. Cotrimoxazole showed a sensitivity of 41.6%. The isolates showed high degrees of resistance to penicillin and cephalosporins. Similarly, the isolates were resistant to fluoroquinolones like ciprofloxacin, having a sensitivity of only 27% (14). In Shariff et al.'s study, the percentage of ciprofloxacin resistance was higher than our results; a ciprofloxacin resistance of 42.2% was reported. Additionally, in Shariff et al.'s study (14), only 0.2% resistance to nitrofurantoin was reported, which was similar to our results.

In this study, a 58.4% resistance rate to TMP-SXT was observed. TMP-SXT has been considered to be the first-line empirical treatment for more than 30 years. The increasing frequency of TMP-SXT resistance is worrisome because this agent is frequently being prescribed for uncomplicated UTIs in many developed and developing countries The resistance of *E. coli* to TMP-SXT is a significant problem in our region, which was demonstrated by other study.

Ihsan Ali's study in 2014 indicated the least bacterial resistance against (%), amikacin (1.8%) (15), tigecycline (2.5%), and nitrofurantoin (3.75%). The resistance rate was 5% for gentamicin, and higher resistances were observed for cefalotin (70%), cefotaxime (58.5%), and ceftazidime (57.5%). Resistance to ciprofloxacin was 57.5%. A high percentage of the isolates in Ihsan Ali's study were resistant to cotrimoxazole (86%) and amoxicillin (76%).

In a 2014 study that tested 50 *E. coli* isolates, Kazemnia showed that all the isolates (100%) were resistant to penicillin and erythromycin, followed by 49 (98%) that were resistant to nalidixic acid, 47 (94%) that were resistant to cephalexin, 43 (86%) that were resistant to amoxicillin, 42 (84%) that were resistant to ampicillin, 37 (74%) that were resistant to ciprofloxacin, 32 (64%) that were resistant to tetracycline, 27 (54%)v to cefixime, and 18 (36%) that were resistant to gentamicin. The results showed that the most effective antibiotic against UPEC isolates was ciprofloxacin (48%) (16). Additionally, most of the isolates were resistant to at least five antibiotics, and MDR was observed in 98% of *E. coli* isolates.

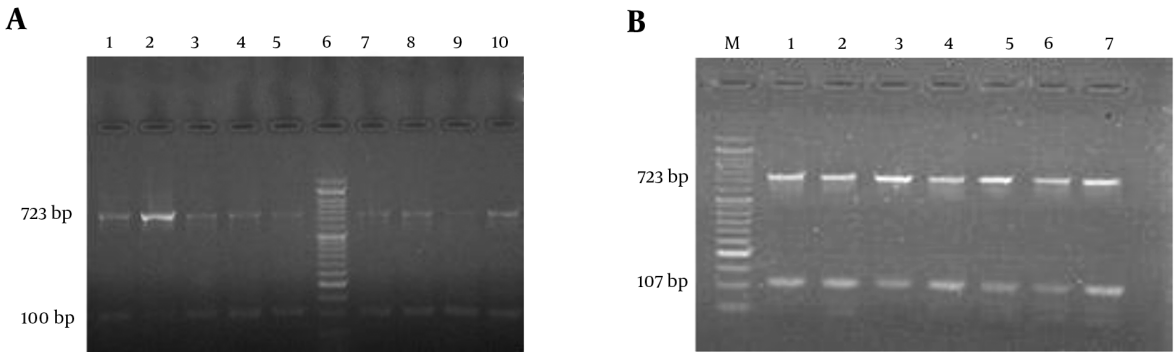

Figure 3. Electrophoresis Analysis of PCR Products of (A) *acrA:* All the bands had a size of 107 bp for the *acrA* gene (lane 10 positive control); (B) *acrB*: All the bands had a size of 107 bp for *acrB* gene (lane 3 positive control). The *16S rRNA* gene had a band size of 723 bp and was the 50-bp ladder in both (A) and (B).

In a study by Asadi et al. in 2014, the rates of resistance to cotrimoxazole, nalidixic acid, ciprofloxacin, cefixime, gentamicin, cephalexin, amikacin, and nitrofurantoin antibiotics were 45%, 41.7%, 21.7%, 20%, 11.7%, 16.7%, 13.3%, and 3.3%, respectively (17). In 2016, Dehbanipour showed that antibiotic resistance to ampicillin, ceftazidime, nalidixic acid, and SXT were higher than 50%. Amikacin, nitrofurantoin, and gentamycin showed markedly greater activity (89.1%, 85.9%, and 82.4% sensitivity, respectively) than other antimicrobial agents (18). MDR is an important clinical bacterial antibiotic resistance that has become a global health concern, threatening the effectiveness of treatment with antibacterial agents. UPEC is one of the most important bacteria that is resistant to several classes of antibiotics, which makes the treatment and improvement of UTIs caused by this bacterium very difficult.

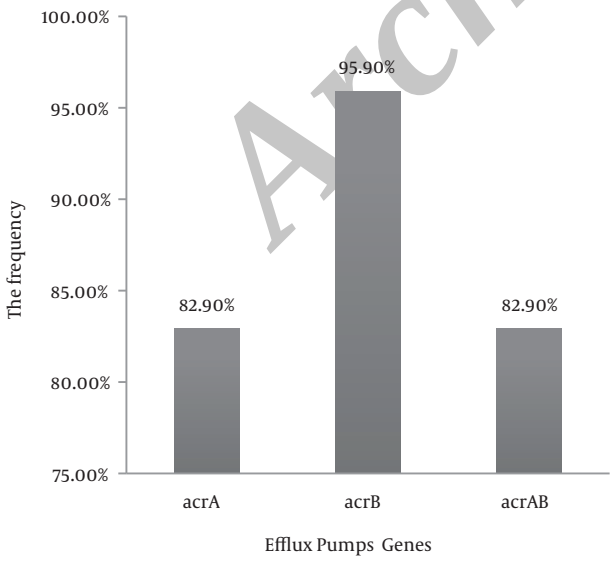

Figure 4. The Frequency of *acrA* and *acrB* Genes Among UPEC Isolates

In this study, 78% of UPEC strains isolated from patients in Milad Hospital were MDR positive and 22% were MDR negative. The highest resistance belonged to 3, 4, 5 and 6 classes of antibiotics. The rates of MDR frequency in our study were different from some studies in other countries. During a 2013 study among community UPEC isolated from 2000 - 2009, Linhares showed the lowest percentage of MDR isolates (17%) (19). The study by Ihsan Ali and his colleagues showed 77.5% prevalence of MDR strains in 2014 in Pakistan (15). The MDR prevalence in Iran, India, Slovaki, and the US have been reported as 77%, 92%, 42% and 7.1%, respectively (15, 20-23). In a study by Dehbanipour in Isfahan, 63% of the studied isolates were found to be resistant to three or more antibiotics (18).

There are many different causes of MDR, and efflux pumps are one of the most important of these causes. Today, true intrinsic resistance of Gram-negative bacteria to antibiotics has been demonstrated to be a result of the high activity of efflux systems, and carrying the efflux pump genes gives bacteria the ability to survive in harsh environments, such as antibiotics (8).

In this study, we evaluated the prevalence of *acrA* and *acrB* genes of efflux pumps in UPEC isolates from patients who were admitted to the Milad hospital in Tehran. The results showed that the frequency of *acrA* and *acrB* genes was 95.5% and 82.9%, respectively. Both genes had the highest frequency (100%) among nitrofurantoin- and amikacin-resistant isolates, but because of the low numbers of these isolates in this study, no significant relationship could be concluded. The lowest prevalence of *acrAB* genes was in gentamicin-resistant bacteria. In 2013, Pakzad showed that all ciprofloxacin-resistant *Klebsiella* isolates harbored the *acrA* gene (24).

Previous studies demonstrated that efflux pumps are able to change the bacterial membrane's permeability for efflux antibiotics out of the cell, leading to decreased intracellular concentrations of antibiotics and resistance to antibiotics. Therefore, either subsequent studies on the increase in the MIC of antibiotics after the expression of *acrAB* genes for different classes of antibiotics should be conducted or the phenotypic activity of efflux pumps should be determined.

According to this study, quinolones are currently a valid option as an experimental therapeutic approach in the treatment of UTLs, but carefully selecting and using these antibiotics is recommended to prevent resistance. Ampicillin is a special agent for treating UTIs caused by of UPEC isolates that cannot be effective in Tehran region. However, nitrofurantoin is still effective for the selective treatment of UTIs caused by UPEC isolates. Additionally, the prevalence of MDR strains among UPEC isolates in this study was very high.

5.1. Conclusions

UTI is the most common infectious disease. As bacterial resistance to the common antimicrobial agents has increased considerably among UPEC isolates, empirical antibiotic treatment should be reviewed periodically at a regional level. Based on findings of this study we should be looking for ways to control the treatment of UTI that do not lead to antibiotic resistance and prevention of MDR strains occurrence. In addition, for better study of efflux pumps, a comprehensive and detailed study regarding the presence and phenotypic and genotypic expression of efflux pumps is required.

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