

Treatment Failure in Urinary Tract Infections: A Warning Witness for Virulent Multi-Drug Resistant ESBL- Producing *Escherichia coli*

This article was published in the following Dove Press journal:
Infection and Drug Resistance

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Background: Global increase in the prevalence of virulent extended-spectrum beta-lactamase (ESBL)-producing uropathogenic *Escherichia coli* (UPEC), which is also multi-drug resistant (MDR), leads to increase in severity of urinary tract infections (UTIs), decrease in the efficacy of the first-line antibiotics, and therefore increase in the morbidity and mortality rates.

Methods: We investigated the distribution of ESBL-producing UPEC in 78 *E. coli* isolates from community-acquired UTI patients in southern Iran. The prevalence of three major ESBL genes, antimicrobial resistance patterns against 15 conventional antibiotic disks, and the presence of 11 important virulence genes that involve in the development and progression of UTIs were evaluated in these isolates.

Results: Of the UPECs, 34.6% were ESBL-positive and 96.3% of the ESBL-producers were MDR. Among the ESBL-producers, 100% harbored *bla*_{CTX-M}, 63% harbored *bla*_{SHV}, and 11.1% harbored *bla*_{TEM} genes. ESBL-producers showed a higher level of resistance to the tested cephalosporins, fluoroquinolones, trimethoprim-sulfamethoxazole, and tetracycline than non-ESBL producers. All isolates were resistant to the tested penicillins. Prevalence of resistance to about two-third of the tested antibiotics was higher than 50% and 93.6% of the isolates were MDR. High prevalence of virulence factors particularly the adhesins (82.1% *csgA*, 73.1% *fimH* genes) and siderophore (73.1% *sitA* gene) was seen in the UPECs. But fortunately in MDR isolates, the virulence score and prevalence of hemagglutinin (*tsh*), hemolysin toxin (*hlyD*) and invasins (*ibeA*) genes were lower than in non-MDR UPECs. Shockingly, among the 15 common antibiotics, only nitrofurantoin (<20% resistance) could be recommended as an appropriate drug for the treatment of UTIs due to our ESBL-producer UPECs.

Conclusion: The alarming level of virulent MDR ESBL-producer *E. coli* strains in this study necessitates the performing of an antibiotic stewardship program, regional screening of ESBL-producers and their virulence properties to select appropriate antibiotic, or designing new therapeutic methods for UTIs by inactivation of the essential virulence factors of UPECs.

Keywords: uropathogenic *Escherichia coli*, extended spectrum beta-lactamases, multi-drug resistance, virulence factor, urinary tract infection

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Introduction

Urinary tract infections (UTIs) due to uropathogenic *Escherichia coli* (UPEC) are common infections in the community, especially in female patients.^{1,2} The existence of several virulence factors (VFs) in UPEC isolates could affect the

occurrence and severity of UTIs.³ Some of these VFs include genes encoding adhesins, such as type-1 fimbriae (*fimH*), pyelonephritis-associated pili (*papA*, *papC*), afimbrial adhesins (*afa*), curli fimbriae (*csgA*), and temperature-sensitive hemagglutinin (*tsh*), which have a role in the colonization of bacteria in the urinary tract and initiation of UTIs, genes encoding toxins that have adverse effects on the host and contribute to the pathogenicity of these strains, such as α -hemolysin (*hlyD*), genes encoding invasins, such as *ibeA* which is involved in brain microvascular endothelial cell invasion, genes encoding protectins, such as increased serum survival (*iss*), which protects *E. coli* against the serum bactericidal activity, genes encoding siderophores, such as the iron transporter *sitA*, which assists in the growth of bacteria, and pathogenicity island marker *malX*, which has a role in the transport of glucose and maltose.⁴⁻⁶

The increase of antibiotic resistance is an emergency health issue in the management of UTIs. One of the most commonly used antibiotics for the treatment of UTIs is beta-lactams.⁷ Extended-spectrum beta-lactamases (ESBL) are enzymes produced by certain bacteria including some strains of *E. coli*, and are able to hydrolyze penicillins, first-third generation cephalosporins, and monobactams (aztreonam). Three main groups of ESBL enzymes include TEM, SHV, and CTX-M, which are inhibited by clavulanic acid, tazobactam, or sulbactam.⁷

Simultaneous resistance to other antibiotic classes such as fluoroquinolones, aminoglycosides, trimethoprim-sulfamethoxazole, tetracyclines, and chloramphenicol, which are often encoded in the same plasmids that harbor the ESBL genes, can complicate and limit the therapeutic options for treatment of infections due to ESBL-producing *E. coli*. This may increase the morbidity costs and mortality rate of UTI patients.⁷

Regarding the geographical variation and the changes over time, it is critical to perform periodic regional and global epidemiological studies to find the frequencies and patterns of drug resistance, and also the occurrence of ESBL producers among UPEC isolates. These data could be useful for choosing better therapeutic strategies and for infection control measures and also help to reduce the public health complications due to untreated or recurrent UTIs. So, the present investigation was done for phenotypic and genotypic evaluation of the distribution of ESBL-producing UPEC in isolates from community-acquired UTI patients in southern Iran. In addition, the antimicrobial resistance patterns and the presence of some important VFs that are

involved in the development, progression, and severity of UTIs were evaluated in these isolates.

Materials and Methods

Sample Collection and Isolation of *E. coli*

We designed this study on 78 urinary *E. coli* isolates which were collected during 8 months from midstream urine samples of outpatients suspected to UTIs [This study was approved by the Shiraz Medical University Ethics Committee, Shiraz, Iran. Informed consent was obtained from participating patients]. These patients had been referred to the microbiology laboratory of Shahid Faghihi hospital (Shiraz, Iran) from various regions of southern Iran (one colony from each patient). The isolates were confirmed as *E. coli* by observation of their phenotypic characteristics on Eosin methylene blue agar (Merck, Darmstadt, Germany) and MacConkey agar (Merck), and also by using conventional biochemical tests.⁸ The confirmed isolates were cultured in Tryptic soy broth (Merck) and conserved with 20% glycerol at -70°C .

Phenotypic Identification of ESBL-Producing UPEC

We used the CLSI phenotypic confirmatory test for identification of ESBL-producing isolates.⁹ In this method, discs containing cefotaxime (30 μg) and ceftazidime (30 μg) alone and combination of cefotaxime-clavulanic acid (30/10 μg) (Padtan Teb, Iran) were applied and the isolates which exhibited at least a 5-mm increase in the inhibition zone diameter for the cefotaxime-clavulanic acid disc versus cefotaxime disc alone were considered as ESBL producers.⁹ *Escherichia coli* ATCC[®] 25922 strain was used as quality control.⁹

Molecular Identification of ESBL-Producing UPEC

We used PCR and electrophoresis techniques to determine the presence of three main types of β -lactamase genes including *bla*_{CTX-M}, *bla*_{SHV}, and *bla*_{TEM} in *E. coli* isolates. DNA of isolates was extracted by the boiling method.⁸ The primer sequences and PCR conditions for detection of β -lactamase genes are shown in Table 1.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility against penicillin, ampicillin, amoxicillin, cephalixin, cefixime, ceftriaxone, ceftazidime, cefotaxime, gentamicin, amikacin, ciprofloxacin, levofloxacin,

Table I List of Primers, Expected Amplicon Size, and the PCR Conditions

Genes	Primer Sequences (5' to 3')	Amplicon Size (bp)	PCR Conditions	References
β-lactamase genes				
<i>bla_{CTX-M}</i>	F: accgccgataattcgagat R: gatatcgttggtggtgccata	588	1 cycle of 94°C for 5 min; 32 cycles of 94°C for 30 s, 54°C for 1 min, 72°C for 2 min; 1 cycle of 72°C for 5 min	[11]
<i>bla_{SHV}</i>	F: cactcaaggatgtattgtg R: ttagcgttgccagtgctcg	885	1 cycle of 94°C for 5 min; 30 cycles of 94°C for 1 min, 50.5°C for 1 min, 72°C for 1 min; 1 cycle of 72°C for 6 min	[12]
<i>bla_{TEM}</i>	F: ttcttgaagacgaagggc R: acgctcagtggaacgaaac	1150	1 cycle of 94°C for 5 min; 30 cycles of 94°C for 1 min, 53°C for 45 s, 72°C for 1 min; 1 cycle of 72°C for 7 min	[12]
Virulence genes				
<i>fimH</i>	F: tcgagaacggataagccgtgg R: gcagtcacctgcccctccgta	508	1 cycle of 95°C for 5 min; 30 cycles of 94°C for 30 s, 62°C for 30 s, 68°C for 3 min; 1 cycle of 72°C for 10 min	[13]
<i>papA</i>	F: atggcagtggtgttttggg R: cgtccaccatacgtgctcttc	717	Same as for <i>fimH</i>	[13]
<i>malX</i>	F: ggacatcctgttacagcgcga R: tcgccaccaatcacagccgaac	925	Same as for <i>fimH</i>	[13]
<i>papC</i>	F: gacggctgactgcagggtgtggcg R: atatcctttctgcaggatgcaata	328	1 cycle of 94°C for 5 min; 30 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 1 min; 1 cycle of 72°C for 10 min	[14]
<i>afa</i>	F: gctgggcagcaaaactgataacttc R: catcaagctgtttgtctccgccg	750	Same as for <i>papC</i>	[14]
<i>csgA</i>	F: actctgacttgactattacc R: agatgcagctcgttcaac	200	Same as for <i>papC</i>	[15]
<i>tsh</i>	F: gggaaatgacctgaatgctgg R: ccgctcatcagtcagtaccac	420	1 cycle of 95°C for 5 min; 30 cycles of 94°C for 30 s, 62°C for 45 s, 72°C for 90 s; 1 cycle of 72°C for 10 min	[15]
<i>hlyD</i>	F: ctccggtacgtgaaaaggac R: gccctgattactgaagcctg	904	Same as for <i>tsh</i>	[13]
<i>ibeA</i>	F: aggcaggtgtgcgcgcgtac R: tgggtctccggcaaacatgc	171	Same as for <i>tsh</i>	[13]
<i>sitA</i>	F: agggggcacaactgattctcg R: taccgggccgtttttctgtgc	608	Same as for <i>tsh</i>	[13]
<i>iss</i>	F: gtggcgaaaactagtaaacagc R: cgcctcggggtggataa	760	1 cycle of 94°C for 4 min; 30 cycles of 94°C for 1 min, 58°C for 1 min, 72°C for 1 min; 1 cycle of 72°C for 5 min	[16]

trimethoprim-sulfamethoxazole (TMP-SMX), nitrofurantoin, and tetracycline (Padtan Teb) was evaluated by the Kirby–Bauer disk diffusion method according to the National Committee for Clinical Laboratory Standards Institute (CLSI).⁹ *Escherichia coli* ATCC® 25922 strain was used as quality control.⁹

Multi-drug resistance (MDR) was defined as non-susceptibility to at least one agent in three or more antimicrobial classes.¹⁰

Detection of Virulence Factor

For evaluation of the prevalence of *fimH*, *papA*, *papC*, *afa*, *csgA*, *tsh*, *hlyD*, *sitA*, *ibeA*, *malX*, and *iss* genes in UPECs, we used the genomic DNA of the isolates, which was extracted by the boiling method in the previous step. Three multiplex PCR processes were designed for groups of *fimH*, *papA*, and *malX* genes, *papC*, *afa*, and *csgA* genes, and *tsh*, *hlyD*, *ibeA*, and *sitA* genes. The *iss* gene was amplified by uniplex PCR. The primer sequences and

the PCR conditions for the detection of these virulence genes are shown in Table 1. Finally, all the PCR products were electrophoresed on a 1% agarose gel (Parstous, Iran) which contained Safe Stain (YTA, Iran), and visualized using a UV-transilluminator (UVitec, Cambridge, UK).

Statistical Analysis

Results were analyzed using the *t*-test, Pearson's chi-square test, or Fisher's exact test. A value of $p \leq 0.05$ was regarded as statistically significant (SPSS 16.0, SPSS Inc., Chicago, IL, USA).

Results

The 78 urine samples which contain *E. coli* belonged to 53 (67.9%) female and 25 (32.1%) male patients suffering from community-acquired UTIs.

Phenotypic and Genotypic Identification of ESBL-Producing UPEC

By using the CLSI phenotypic confirmatory test, among the 78 *E. coli* isolates, 27 (34.6%) isolates were detected as ESBL producers. Thus, 14 strains of them belonged to female patients and 13 strains belonged to male patients.

Overall, the prevalences of β -lactamase genes including *bla*_{CTX-M}, *bla*_{SHV}, and *bla*_{TEM} in 78 *E. coli* isolates were 29 (37.2%), 37 (47.4%), and 12 (15.4%), respectively. Based on the gender of the source of UPEC isolates, the prevalence of *bla*_{CTX-M} gene was 16 (30.2%) and 13 (52%), *bla*_{SHV} gene was 25 (47.2%) and 12 (48%), and *bla*_{TEM} was 8 (15.1%) and 4 (16%), for females' and males' isolates, respectively.

All the 27 (100%) UPEC isolates that phenotypically confirmed as ESBL producers harbored *bla*_{CTX-M} gene, 17 (63%) harbored *bla*_{SHV} gene, and 3 (11.1%) harbored *bla*_{TEM} gene.

The distribution of three main types of β -lactamase genes in UPEC isolates that were phenotypically confirmed as ESBL producers revealed four patterns. Two isolates (7.4%) harbored all the three tested ESBL genes, 15 isolates (55.6%) harbored *bla*_{CTX-M} and *bla*_{SHV}, one isolate (3.7%) harbored *bla*_{CTX-M} and *bla*_{TEM}, and 9 isolates (33.3%) only harbored *bla*_{CTX-M} gene.

The prevalence of *bla*_{CTX-M} ($p < 0.001$) and *bla*_{SHV} ($p = 0.04$) genes was significantly higher in ESBL producers than non-ESBL producers, but for *bla*_{TEM} gene prevalence no significant difference ($p > 0.05$) was found in these two groups of isolates. The prevalence of *bla*_{CTX-M},

*bla*_{SHV}, and *bla*_{TEM} in MDR and non-MDR isolates was not significantly different ($p > 0.05$).

Antimicrobial Susceptibility Testing

Results of antimicrobial susceptibility testing against 15 antimicrobial agents showed that all 78 (100%) UPEC isolates were resistant to penicillins (penicillin, ampicillin, and amoxicillin). The lowest prevalence of resistance was seen against aminoglycosides (16.7% to gentamicin, 21.8% to amikacin) and then nitrofurantoin (23.1%) (Table 2). Also, 2/78 (2.6%) of UPEC isolates were resistant to all 15 tested antimicrobial agents and 41/78 (52.6%) of UPEC isolates were resistant to at least half (≥ 8) of the tested antimicrobial agents. Also, four (5.1%) UPEC isolates only show resistance against the tested penicillins.

Overall, 53 different resistance patterns were found among the 78 tested isolates, of which the resistance pattern of penicillin, ampicillin, amoxicillin, cephalixin, cefixime, ceftriaxone, ceftazidime, cefotaxime, ciprofloxacin, levofloxacin, trimethoprim-sulfamethoxazole, and tetracycline that was seen in six UPEC isolates was the predominant pattern.

Approximately 93.6% (73/78) of the UPEC isolates were multi-drug resistant (MDR). So that the 94.3% (50/53) of females' isolates and 92% (23/25) of males' were MDR. Also, the frequency of MDR in ESBL-producer isolates was 96.3% (26/27). The prevalence of MDR was not significantly different in ESBL-producer and non-ESBL producer isolates ($p > 0.05$).

The prevalence of antimicrobial resistance against the 15 tested antimicrobial agents in all UPEC isolates, and also separately in MDR and non-MDR, ESBL producers and non-ESBL producers, females' and males' isolates, and isolates which harbored each of the tested *bla* genes is showed in Table 2.

Comparatively, antimicrobial resistance against cephalixin ($p = 0.03$), ceftriaxone ($p = 0.03$), ciprofloxacin ($p = 0.007$), levofloxacin ($p = 0.009$), trimethoprim-sulfamethoxazole ($p = 0.04$), and tetracycline ($p = 0.006$) was more prevalent in MDR isolates than non-MDR isolates. However, for resistance against other tested antibiotics, no significant differences were seen in these two groups of isolates ($p > 0.05$) (Table 2).

Antimicrobial resistance against the tested cephalosporins ($p < 0.001$), Fluoroquinolones ($p < 0.05$), trimethoprim-sulfamethoxazole ($p = 0.02$), and tetracycline ($p = 0.04$) was higher in ESBL-producer isolates than non-ESBL producers, but for the tested penicillins, aminoglycosides, and

Table 2 Prevalence of the Antimicrobial Resistance Among the UPEC Isolates^a

Antibiotics	Resistance in	Resistance in		Resistance in		Resistance in		Resistance in		
	All Isolates (n=78)	MDR Isolates (n=73)	Non- MDR Isolates (n=5)	ESBL Producers (n=27)	Non- ESBL Producers (n=51)	Females' Isolates (n=53)	Males' Isolates (n=25)	<i>bla</i> _{CTX-M} Positive Isolates (n=29)	<i>bla</i> _{SHV} Positive Isolates (n=37)	<i>bla</i> _{TEM} Positive Isolates (n=12)
β- lactam antibiotics (penicillins)										
Penicillin	78 (100)	73 (100)	5 (100)	27 (100)	51 (100)	53 (100)	25 (100)	29 (100)	37 (100)	12 (100)
Ampicillin	78 (100)	73 (100)	5 (100)	27 (100)	51 (100)	53 (100)	25 (100)	29 (100)	37 (100)	12 (100)
Amoxicillin	78 (100)	73 (100)	5 (100)	27 (100)	51 (100)	53 (100)	25 (100)	29 (100)	37 (100)	12 (100)
β- lactam antibiotics (first generation cephalosporins)										
Cephalexin	44 (56.4)	43 (58.9)	1 (20.0)	23 (85.2)	21 (41.2)	26 (49.1)	18 (72.0)	25 (86.2)	23 (62.2)	8 (66.7)
β- lactam antibiotics (third generation cephalosporins)										
Cefixime	43 (55.1)	42 (57.5)	1 (20.0)	24 (88.9)	19 (37.3)	25 (47.2)	18 (72.0)	26 (89.7)	24 (64.9)	7 (58.3)
Ceftriaxone	42 (53.8)	41 (56.2)	1 (20.0)	26 (96.3)	16 (31.4)	24 (45.3)	18 (72.0)	28 (96.6)	22 (59.5)	7 (58.3)
Ceftazidime	34 (43.6)	34 (46.6)	0 (0.0)	20 (74.1)	14 (27.5)	17 (32.1)	17 (68.0)	21 (72.4)	18 (48.6)	6 (50.0)
Cefotaxime	43 (55.1)	42 (57.5)	1 (20.0)	27 (100)	16 (31.4)	25 (47.2)	18 (72.0)	29 (100)	24 (64.9)	6 (50.0)
Aminoglycosides										
Gentamicin	13 (16.7)	12 (16.4)	1 (20.0)	7 (25.9)	6 (11.8)	4 (7.5)	9 (36.0)	8 (27.6)	5 (13.5)	3 (25.0)
Amikacin	17 (21.8)	17 (23.3)	0 (0.0)	8 (29.6)	9 (17.6)	11 (20.8)	6 (24.0)	8 (27.6)	7 (18.9)	4 (33.3)
Fluoroquinolones										
Ciprofloxacin	47 (60.3)	47 (64.4)	0 (0.0)	21 (77.8)	26 (51.0)	28 (52.8)	19 (76.0)	23 (79.3)	22 (59.5)	7 (58.3)
Levofloxacin	44 (56.4)	44 (60.3)	0 (0.0)	20 (74.1)	24 (47.1)	25 (47.2)	19 (76.0)	22 (75.9)	21 (56.8)	7 (58.3)
Dihydrofolate reductase inhibitors										
Trimethoprim-Sulfamethoxazole	50 (64.1)	49 (67.1)	1 (20.0)	22 (81.5)	28 (54.9)	31 (58.5)	19 (76.0)	24 (82.8)	26 (70.3)	9 (75.0)
Nitrofurans										
Nitrofurantoin	18 (23.1)	18 (24.7)	0 (0.0)	5 (18.5)	13 (25.5)	11 (20.8)	7 (28.0)	6 (20.7)	6 (16.2)	1 (8.3)
Tetracyclines										
Tetracycline	49 (62.8)	49 (67.1)	0 (0.0)	21 (77.8)	28 (54.9)	29 (54.7)	20 (80.0)	22 (75.9)	24 (64.9)	8 (66.7)

Note: ^aValues were showed as No. (%). The highest and lowest amounts in each column are emboldened.

nitrofurantoin, the prevalence of resistance was not significantly different in these two groups of isolates ($p>0.05$) (Table 2).

Furthermore, the frequencies of antimicrobial resistance against cefotaxime ($p=0.04$), gentamicin ($p=0.003$), levofloxacin ($p=0.04$), and tetracycline ($p=0.03$) were higher in males' isolates than females'; however, resistance to other tested antibiotics was not significantly different in females' and males' isolates (Table 2).

Statistical analysis showed significant correlations between resistance to ciprofloxacin ($p=0.01$),

levofloxacin ($p=0.02$), trimethoprim-sulfamethoxazole ($p=0.007$), and all the tested cephalosporins ($p<0.001$) and presence of the *bla*_{CTX-M} gene in the UPEC isolates. The presence of the *bla*_{SHV} gene also contributed to the high prevalence of resistance to cefotaxime ($p=0.04$). But resistance to nitrofurantoin was more prevalent in isolates which did not harbor the *bla*_{SHV} gene ($p=0.02$). No significant association was found between resistance to the tested antibiotics and the presence of the *bla*_{TEM} gene ($p>0.05$) (Table 2).

The resistance score ranges, means, and medians in different groups of UPEC isolates are demonstrated in Table 3.

Table 3 Resistance and Virulence Scores Among the UPEC Isolates^a

Scores		In		In		In		In		In		In		In	
		All Isolates (n=78)	MDR Isolates (n=73)	Non-MDR Isolates (n=5)	ESBL Producers (n=27)	Non-ESBL Producers (n=51)	Females' Isolates (n=53)	Males' Isolates (n=25)	bla _{CTX-M} Positive Isolates (n=29)	bla _{CTX-M} Negative Isolates (n=49)	bla _{SHV} Positive Isolates (n=37)	bla _{SHV} Negative Isolates (n=41)	bla _{TEM} Positive Isolates (n=12)	bla _{TEM} negative isolates (n=66)	
Resistance score															
Range	3–15	3–15	3–7	4–15	3–14	3–14	3–15	4–15	3–14	3–15	3–15	4–14	3–15		
Mean	8.7	9.0	4.2	11.3	7.3	7.8	10.5	11.3	7.1	9.0	8.4	9.1	8.6		
Median	8.5	9.0	4.0	12.0	7.0	7.0	12.0	12.0	6.0	9.0	7.0	9.0	8.5		
Virulence score															
Range	1–10	1–9	5–10	1–7	1–10	1–9	1–10	1–7	1–10	1–9	1–10	1–6	1–10		
Mean	4.5	4.3	7.2	4.4	4.5	4.5	4.4	4.4	4.5	4.4	4.5	3.8	4.6		
Median	4.5	4.0	6.0	5.0	4.0	4.0	5.0	5.0	4.0	5.0	4.0	4.5	4.5		

Note: ^aThe highest and lowest amounts in each row are emboldened.

The resistance score in ESBL producers ($p<0.001$), MDR isolates ($p=0.003$), males' isolates ($p=0.002$), and isolates which harbored the *bla*_{CTX-M} gene ($p<0.001$) were significantly higher than in other comparable isolates (Table 3).

Detection of Virulence Factor

Among the 11 examined VFs, the highest prevalence was detected for *csgA* (82.1%), *fimH* (73.1%), and *sitA* (73.1%) genes, respectively. The prevalence of other VFs in the UPEC isolates was below 50%. The *afa* and *ibeA* genes with 14.1% frequency had the lowest prevalence (Table 4).

One (1.3%) of the UPEC isolates harbored 10 out of the 11 tested VFs and only lacked the *papA* gene. Of the UPEC isolates, 23/78 (29.5%) harbored at least half (≥ 6) of the tested VFs; three isolates only had *csgA*, two isolates only had *fimH*, and one isolate only had the *sitA* gene.

Prevalences of the tested VFs in all UPEC isolates, and also separately in MDR and non-MDR, ESBL producers and non-ESBL producers, females' and males' isolates, and isolates harboring each of the tested *bla* genes are showed in Table 4.

In the ESBL producer and non-ESBL producer isolates, the prevalence of none of the tested VFs was significantly different ($p>0.05$). But fortunately in the MDR UPECs with limited inhibitory options, prevalence of *tsh* ($p=0.05$), *hlyD* ($p=0.01$) and *ibeA* ($p=0.02$) genes were lower than in the non-MDR UPECs. Furthermore, the prevalence of the tested VFs in females' and males' UPEC isolates was not significantly different ($p>0.05$), except for the *csgA* gene which was more prevalent in males' isolates ($p=0.03$), and the *sitA* gene which was more prevalent in females' isolates ($p=0.02$). Also, higher *bla*_{SHV} gene was found in the isolates which had the *papA* gene than the isolates which lack this gene ($p=0.04$). The prevalence of the *bla*_{CTX-M} gene was higher in isolates which did not have the *ibeA* gene than the isolates which carried this gene ($p=0.04$). For other genes, no significant association was found ($p>0.05$) (Table 4).

A total of 65 virulence patterns were found among the isolates, among which the virulence pattern of “*fimH*, *csgA*” that was seen in four UPEC isolates was the pre-dominant pattern.

The virulence score ranges, means, and medians in different groups of UPEC isolates are demonstrated in Table 3. The virulence score in non-MDR isolates was higher than in MDR isolates ($p=0.002$). But no significant

Table 4 Prevalence of the Virulence Genes Among the UPEC Isolates^a

Virulence Gene	Prevalence in	Prevalence in		Prevalence in		Prevalence in		Prevalence in		
	All Isolates (n=78)	MDR Isolates (n=73)	Non-MDR Isolates (n=5)	ESBL Producers (n=27)	Non-ESBL Producers (n=51)	Females' Isolates (n=53)	Males' Isolates (n=25)	<i>bla</i> _{CTX-M} Positive Isolates (n=29)	<i>bla</i> _{SHV} Positive Isolates (n=37)	<i>bla</i> _{TEM} Positive Isolates (n=12)
Adhesin										
<i>fimH</i>	57 (73.1)	52 (71.2)	5 (100)	18 (66.7)	39 (76.5)	41 (77.4)	16 (64.0)	20 (69.0)	27 (73.0)	9 (75.0)
<i>papA</i>	27 (34.6)	24 (32.9)	3 (60.0)	10 (37.0)	17 (33.3)	19 (35.8)	8 (32.0)	12 (41.4)	17 (45.9)	2 (16.7)
<i>papC</i>	35 (44.9)	32 (43.8)	3 (60.0)	16 (59.3)	19 (37.3)	23 (43.4)	12 (48.0)	16 (55.2)	15 (40.5)	3 (25.0)
<i>csgA</i>	64 (82.1)	60 (82.2)	4 (80.0)	23 (85.2)	41 (80.4)	40 (75.5)	24 (96.0)	25 (86.2)	29 (78.4)	9 (75.0)
<i>afa</i>	11 (14.1)	10 (13.7)	1 (20.0)	4 (14.8)	7 (13.7)	8 (15.1)	3 (12.0)	4 (13.8)	4 (10.8)	1 (8.3)
<i>tsh</i>	28 (35.9)	24 (32.9)	4 (80.0)	8 (29.6)	20 (39.2)	20 (37.7)	8 (32.0)	8 (27.6)	14 (37.8)	3 (25.0)
Toxin										
<i>hlyD</i>	19 (24.4)	15 (20.5)	4 (80.0)	6 (22.2)	13 (25.5)	12 (22.6)	7 (28.0)	7 (24.1)	7 (18.9)	2 (16.7)
Invasin										
<i>ibeA</i>	11 (14.1)	8 (11.0)	3 (60.0)	1 (3.7)	10 (19.6)	7 (13.2)	4 (16.0)	1 (3.4)	3 (8.1)	4 (33.3)
Protectin										
<i>iss</i>	18 (23.1)	16 (21.9)	2 (40.0)	4 (14.8)	14 (27.5)	10 (18.9)	8 (32.0)	4 (13.8)	7 (18.9)	0 (0.0)
Siderophore										
<i>sitA</i>	57 (73.1)	52 (71.2)	5 (100)	19 (70.4)	38 (74.5)	43 (81.1)	14 (56.0)	21 (72.4)	29 (78.4)	10 (83.3)
Miscellaneous										
<i>malX</i>	21 (26.9)	19 (26.0)	2 (40.0)	9 (33.3)	12 (23.5)	16 (30.2)	5 (20.0)	10 (34.5)	10 (27.0)	2 (16.7)

Note: ^aValues were showed as No. (%). The highest and lowest amounts in each column are emboldened.

difference was found for the virulence score of ESBL and non-ESBL producers, males' and females' isolates, and isolates with or without each of the tested *bla* genes ($p>0.05$) (Table 3).

The associations between the antibiotic resistance and presence of the virulence factors and also the associations of the presence of the virulence genes with each other are shown in [Supplementary Materials](#) and [Tables S1–S4](#).

Discussion

In the recent decade, there has been universal concern about the management of UTIs caused by *E. coli*. The worldwide increase in the prevalence of virulent ESBL-producing UPEC which are also MDR has led to increased severity of UTIs, decreased the efficacy of first-line antibiotics, and therefore increased medical costs and the rate of morbidity and mortality. Accurate global estimation of these problems is very difficult, since according to the World Health Organization (WHO) global report on surveillance of antimicrobial resistance there was not enough information about the worldwide extent of the antimicrobial resistance, particularly in developing countries.¹⁷ So

we conducted the present research to find the effective drug to combat *E. coli* infections on the basis of the regional rate and pattern of antimicrobial resistance and also to prognose the severity of UTIs by the evaluation of essential VFs in the UPEC isolates.

During the period of sampling, frequencies of UTIs and isolation of *E. coli* from urine samples of female patients were higher (more than twice) than those of the males, which is in agreement with the previous reports in Iran and other countries.^{18–21} This may be due to the difference in the anatomical structure of the females' and males' genital tract, as comparatively the females' urethra is shorter and therefore infectious agents (eg, *E. coli*) can more easily reach the bladder. In addition, the position of the females' urethral opening near the anus and vagina also plays a role in easier entry of the bacteria from these sites to the urinary tract system.¹⁹

ESBL-Producing UPEC

The β -lactam antibiotics were mainly used for the treatment of infections due to *E. coli* strains, but recently the worldwide increase of the ESBL-producing *E. coli* is a major concern of clinicians.⁷ Lee et al²² in 2018 reviewed and reported that the

prevalence of ESBL-producing *E. coli* isolates was increased in UK (from 4.6% to 6.6%), France (from 1.1% to 3.3%), Spain (from 2.4–18.2% to 8.9–23.6%), Italy (from 3.5% to 6.7%), Turkey (from 8.0–13.1% to 24.0%), South Asia (from 21.7% to 33.2%), Far East Asia (from 4.8–7.5% to 7.6–10.7%), Latin America (from 1.7% to 7.1–12.5%), USA and Canada (from 7.4% to 1.8–8.0%) before and after 2010.

In our study, 34.6% of the UPEC isolates were ESBL producers. As a result, it can be said that common β -lactam drugs could not effect at least one-third of these UPECs. In another study²³ on *E. coli* isolates from UTIs in Kerman, Iran, 41% of outpatients had ESBL-positive isolates. A somewhat similar (40%) rate of ESBL producers was reported in UPECs in a study in Pakistan.²⁰ This high prevalence of ESBL-producer *E. coli* in Iran and other developing countries in contrast with developed countries may be a result of ample use of β -lactam drugs and the absence of a strict policy for consumption and even prescription of the antibiotics in these regions.

Unlike the lower incidence of UTIs in males, the UPEC isolates from male patients were higher ESBL producers than those from females (52% versus 26.4%) ($p=0.03$). But the differences in the prevalence of *bla*_{CTX-M}, *bla*_{SHV}, and *bla*_{TEM} genes in females' and males' isolates were not significant ($p>0.05$). Similarly, in a study from Spain,²⁴ a significant association was found between UTIs due to ESBL-positive *E. coli* and male sex. In contrast, in another study in Iran,²³ the production of ESBLs in UPEC isolates of different gender was not significantly different.

Since several studies revealed that the ESBL producers also show a high level of resistance to non- β -lactam antibiotics, the global concerns about ESBL-producing *E. coli* are increasing. As in our study, antimicrobial resistance against the tested cephalosporins ($p<0.001$), fluoroquinolones ($p<0.05$), trimethoprim-sulfamethoxazole ($p=0.02$), and tetracycline ($p=0.04$) were higher in ESBL-producer isolates than non-ESBL producers. Similarly, in a study by Azap et al²⁵ on UPEC isolates from community-acquired UTIs in Turkey, resistance to ciprofloxacin and trimethoprim-sulfamethoxazole in ESBL producers was higher than in non-ESBL producers ($p<0.001$). In their study,²⁵ the gentamicin resistance was also higher in ESBL-producer isolates than non-ESBL producers (57% vs 9%) ($p<0.001$). But in our study, although the rate of gentamicin resistance in ESBL-producers was higher than in non-ESBL producers (25.9% vs 11.8%), this difference is not

statistically significant ($p>0.05$). Additionally, in a study from Spain,²⁶ the prevalence of resistance to ciprofloxacin was 31.5% vs 9.1% in ESBL-producer and non-ESBL-producer *E. coli* isolated from community-acquired UTIs.

In ESBL-positive isolates, resistance to nitrofurantoin had the lowest prevalence (18.5%). A low nitrofurantoin resistance rate also reported in other studies.^{19,25} Lee et al²² declared a 90% sensitivity to nitrofurantoin in *E. coli* isolates from community-acquired UTIs in most countries around the world. Therefore, the European Association of Urology (EAU) and Infectious Diseases Society of America (IDSA) recommended nitrofurantoin as the first-line treatment for UTIs, if the regional prevalence of resistance to this drug is below 20%.²² This drug was also suggested as the first-line treatment option for UTIs in other studies from Iran and other countries.^{2,19,20,22,27} In the study by Derakhshandeh et al,²⁸ resistance against nitrofurantoin (12.9%) had the lowest frequency compared to the other tested antibiotics. But a comparison of our results with their study, which was conducted in the same area as ours, revealed that from 2012 to the present the rates of resistance to nitrofurantoin have increased in this area. This could be due to a recent increase in the prescription of this antibiotic for the empirical treatment of UTIs in this area.

The rates and order of prevalence of β -lactamase genes were different in various studies from distinct and even similar regions, which could indicate their epidemiological diversity.^{21,29,30}

In the present study, all (100%) of the ESBL producers harbored cefotaximase (*bla*_{CTX-M}) gene. Confirming this result, all (100%) of the ESBL-producer isolates were resistant to cefotaxime. Likewise, 100% of ESBL producers in a study from Bolivia,²⁷ 99.5% of ESBL-positive *E. coli* in a study from western Saudi Arabia,³⁰ and 98.0% of ESBL-positive UPECs from community-acquired UTIs in the study from Turkey²⁵ were found to harbor the *bla*_{CTX-M} gene. This revealed that the *bla*_{CTX-M} type is the most frequent ESBL gene in different regions around the world.

The majority (66.7%) of ESBL-producer *E. coli* isolates harbored more than one ESBL gene. The combination of *bla*_{CTX-M} and *bla*_{SHV} (55.6%) was the most common pattern for β -lactamase genes. A combination of all the three tested ESBL genes was detected in 7.4% of the UPECs. In contrast, in a study from western Saudi Arabia,³⁰ 82.5% of *E. coli* isolates contained more than one ESBL gene; the frequent ESBL gene pattern in ESBL producers was a combination of *bla*_{CTX-M} and *bla*_{TEM}

(79.1%); and a combination of CTX-M, TEM, and SHV types of β -lactamase genes was detected in only 2.4% of *E. coli* isolates.

Antimicrobial Resistance

In the present study, the overall rates of antimicrobial resistance were relatively high, as the prevalence of resistance to 11/15 (73.3%) of the tested antibiotics was higher than 50%. Furthermore, all (100%) of the isolates were resistant to penicillin, ampicillin, and amoxicillin, and the lowest prevalence of resistance was seen against aminoglycosides (16.7% to gentamicin, 21.8% to amikacin). These results are consistent with the results of a similar study in Mexico⁵ which reported that 97.4% of *E. coli* isolates were resistant to ampicillin, and the lowest rate of resistance was seen against amikacin (14.4%). Also, in a study from western Saudi Arabia,³⁰ the rate of ampicillin resistance in *E. coli* isolates was 99.5% and the lowest antibiotic resistance was reported against amikacin. So, because of the increase in the prevalence of resistance to the penicillins, these antibiotics are not currently recommended for empiric therapy of UTIs.

Following the resistance to penicillins, most of our UPECs were resistant to trimethoprim-sulfamethoxazole (64.1%), which is a drug of choice for treatment of UTIs. Ali et al²⁰ also reported the high (82%) prevalence of trimethoprim-sulfamethoxazole resistance in UPECs isolated in Pakistan. In a study from Mexico,²¹ more than 70% of UPECs were resistant to trimethoprim-sulfamethoxazole. In another study in Mexico,⁵ this prevalence was 66%. Additionally, Sanchez et al³¹ found a great increase in the rate of resistance to trimethoprim-sulfamethoxazole in urinary *E. coli* isolates among US outpatients from 2000 to 2010. So, the application of this antibiotic in the treatment of UTIs may become limited.

Since fluoroquinolones are frequently recommended for the treatment of uncomplicated pyelonephritis and complicated UTIs, the resistance of UPECs to these antibiotics is very important. In our study, more than half of the isolates were fluoroquinolone-resistant and all the fluoroquinolone-resistant strains showed MDR phenotype. This was comparable to the results of studies in North America³² and Mexico.²¹ The increased resistance against fluoroquinolones may be due to the excessive use of these antibiotics which could restrict their recommendation for the treatment of UPEC infections.

In our UPECs, the mean of resistance rates against the tested cephalosporins was above 50%. Relatively similar

or different antimicrobial resistance rates have been determined in UPEC isolates from other regions of Iran^{19,23,33} and also other countries including Pakistan,²⁰ Turkey,²⁵ Mexico,^{5,21} Saudi Arabia,³⁰ Brazil,³⁴ Mongolia,² etc.²² Therefore, without the regional and even case by case evaluation of antimicrobial susceptibilities of infecting *E. coli* should not use the cephalosporins as the empirical drugs for the treatment of UTIs.

Unfortunately, we found a high level (93.6%) of MDR *E. coli* isolates in this study. This result may be due to easy access to the common antimicrobial drugs without prescription in our region. This serious public health problem could strongly limit the choice of antibiotics for the treatment of UTIs due to *E. coli*.

A high prevalence of MDR *E. coli* was also reported in relative studies in China,³⁵ western Saudi Arabia,³⁰ Mexico,⁵ and Mongolia² (100%, 100%, 96.9%, and 93.9%, respectively). Due to the rules and prescribing behaviors in different regions, the rate of MDR isolates was varied in different countries. For example, in studies from Turkey,²⁵ Spain,²⁶ Mexico,²¹ and Pakistan,²⁰ the rates of MDR *E. coli* were 70.6%, 70%, 63%, and 59%, respectively. However, in a study from the Kerman province of Iran, 55.6% of UPECs from outpatients were reported as MDR isolates.²³ In comparison with a previous study²⁸ in the same area as our study (Shiraz, Iran), the MDR rate in UPEC isolates of outpatients with UTIs has increased from 82.3% to 93.6% during 2012 to the present study.

In contrast with the results of the study in Mexico,²¹ which reported that the UPECs from male patients were associated with the MDR phenotype, in our study the prevalence of MDR in females' and males' UPEC isolates was not significantly different ($p>0.05$). However, the resistance score was higher in males' isolates than females' ($p=0.002$). Differences in the rates of resistance against certain antibiotics in isolates of males and females may be due to differences in the pattern of antibiotic consumption. For example, similar to our study, in the studies in Pakistan²⁰ and Mexico,²¹ fluoroquinolone-resistant UPECs were significantly more prevalent among male patients than females ($p<0.05$). That could be because of using fluoroquinolones as a first choice for the treatment of UTIs in men. This may lead to an increase in the UTI treatment failure in male patients.

Virulence Factors

The presence of multiple VFs in the UPECs could indicate the high level of their pathogenicity. Furthermore, the

presence of MDR and expression of the beta-lactamase genes in more virulent bacteria also could restrict the practical use of antibiotics for the treatment of their infections.

In agreement with the study³⁶ in Tehran, Iran, all isolates in our study harbored at least one of the tested VFs. However, in other studies from Iran^{19,33} and also in two studies from Brazil,^{34,37} 41.1%, 43.2%, 10%, and 51% of UPECs had none of the tested VFs, respectively. These results could show a difference in the number and distribution of the VFs among UPEC strains of various geographical regions.³⁶

An essential stage for UTI development is the adhesion of pathogenic *E. coli* to different parts of the urinary tract system. Thereafter, UPECs can colonize, invade, and replicate within the uroepithelial cells.⁵ That is why the high frequencies of adhesin-encoding genes were detected in the tested UPEC isolates. The *csgA* (82.1%) and *fimH* (73.1%) genes were the most prevalent adhesion genes in our UPEC isolates, followed by *papC* (44.9%), and *afa* gene had the lowest prevalence (14.1%) among the adhesin genes. The *csgA* gene has a main role in biofilm formation and also can function in adhesion processes in human cystitis. The *fimH* gene, which encodes the type 1 fimbriae, has a role in bacterial survival, induction of inflammation, invasion, and mediating bacterial colonization and biofilm formation in the murine UTI model, but as expressed in both pathogenic and commensal *E. coli*, their role in the pathology of human UTIs is unclear. P fimbriae and the *papC* gene have a principal role in the pathophysiology of pyelonephritis due to UPECs, and finally the *afa* gene is involved in chronic and recurrent UTIs.^{3,38,39}

In a study³⁸ from Brazil, the prevalence of the *csgA* gene in 31 *E. coli* isolates from recurrent cystitis was 100%, and those of *fimH* and *papC* were 87.1% and 25.8%, respectively. Similarly, in a study from Mexico,²¹ the *afa* gene had the lowest distribution (8.2%) among the UPEC isolates and the prevalences of *fimH* and *papC* were 73.4% and 40.5%, respectively. Also, in a study from Tunisia,³ *afa* (20.0%) had the lowest prevalence among the tested adhesion genes and the prevalences of *fimH* and *pap* were 60.0% and 28.0%, respectively. In another study from Mexico,⁵ *iha* (64.9%) followed by *fim* (61.3%) and *pap* (24.7%), were the most frequent adhesion genes in the UPEC isolates. In a study⁴⁰ from Karaj, Iran, the fimbriae type 1 (*fimH*; 73.0%) and P fimbriae (*pap*; 46.0%) were the most prevalent adhesins. The difference in the

percentage of individual adhesin genes in various studies may be because of the presence of a variety of adhesins which UPECs can use for binding to the uroepithelial cells.

In addition to adhesion, iron acquisition is critical for the pathogenesis of UPECs. In our isolates subsequent to the *csgA* gene and equal to the *fimH* gene, the *sitA* gene (73.1%) had the highest prevalence among the other tested VFs. This may reveal that the majority of our UPECs could survive in an iron-limited urinary tract system, overcome the host defense mechanism, and develop the UTIs.⁴¹ Concerning the other VFs, the *ibeA* (14.1%) invasion gene had the lowest prevalence among the tested UPECs, which supports the results of the previous study²⁸ in the same area.

Overall, 24.4% of our isolates harbored the *hlyD* gene, which indicated that these strains could produce α -hemolysin toxin potentially. Hemolysin has roles in the pathogenesis of UPECs, causes inflammation and permanent renal scarring and contributes to the severity and development of cystitis.³⁹ The prevalence of the hemolysin gene in UPEC isolates was 17.6% in a study from Kurdistan, Iran;¹⁹ 21.2% in a study from Shiraz, Iran;²⁸ 47.0% in a study from Karaj, Iran;⁴⁰ 13% in a study from Tunisia,³ and 9.1% in a study from Mexico.²¹

In previous studies^{28,33} in the same area as our study (Shiraz, Iran), UPECs which were isolated from UTI outpatients in 2012 harbored lower rates of *fimH* (34.1%), *papA* (9.4%), *hlyD* (21.2%), *sitA* (15.3%), *tsh* (27.1%), *iss* (20.0%), *malX* (16.5%), and *ibeA* (9.4%) genes than our UPECs, which could indicate the increase of the VFs in UPEC isolates during this period in this area. Relatively similar or different prevalences of VFs have been determined in UPEC isolates from other regions of Iran¹⁹ and also other countries including Tunisia,³ Brazil,³⁴ and Mongolia.²

In agreement with our study, in another study from Iran¹⁹ the prevalence of most tested VFs in females' and males' UPEC isolates was not significantly different ($p>0.05$). In contrast, the results of the study from Mexico²¹ indicated that the females' isolates had a higher mean of VFs compared to those from males'.

Conclusion

Alarmingly, in the present study more than one-third of the UPECs were ESBL-positive and almost all of the ESBL-producer strains were MDR. These strains are a crucial problem for public health because they can play a role in the dissemination of antimicrobial resistance in the

community and cause most of the commonly used antibiotics for treatment of UTIs to become ineffective in the near future in this area. Shockingly, among the 15 common antibiotics, only nitrofurantoin could be recommended as an appropriate drug for the treatment of UTIs due to our ESBL-producer UPECs. Overall, due to differences in the characteristics of *E. coli* strains from various geographical regions and their alterations over time, regular regional screening of ESBL producers and their virulence properties is necessary for selecting the appropriate antibiotic or designing new therapeutic methods for UTIs, especially in developing countries.

Acknowledgments

This work was supported by Shiraz University [Grant number 96GRD1M344385]. We acknowledge Dr. Mohammad Motamedifar for helping in sample collection and also we thank Nafiseh Karimi, Azar Ghaedi, and Bahare Behzadnia for their contribution to a part of this research.

Disclosure

The authors declare that they have no conflicts of interest in this work.

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