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Phylogenetic Grouping and Assessment of Virulence Genotypes, With Antibiotic Resistance Patterns, of *Escherichia Coli* Strains Implicated in Female Urinary Tract Infections

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Abstract

Background: *Escherichia coli* strains are common pathogens that can cause urinary tract infections (UTIs). They are classified into phylogroups based on three genetic markers: *chuA*, *yjaA*, and *TspE4.C2*. The *E. coli* strains that cause UTIs possess several genes that encode urovirulent factors and antimicrobial-resistance phenotypes. We determined the phylogenetic groups of *E. coli* isolates from UTI cases in Sabzevar, Iran, the prevalence of certain virulence genes, and the antibiotic-resistance phenotypes in these strains.

Objectives: The aim of this study was to assess the correlation of detected *E. coli* phylogroups in female UTI patients with the antibiotic-resistance pattern and the distribution of certain virulence factors among the phylogroups.

Materials and Methods: Ninety-three *E. coli* isolates from 150 women with UTI were studied. Three genetic markers were detected for phylogenetic grouping of strains, and four virulence determinants were analyzed with multiplex-PCR, including the genes for hemolysin (*hly*), *aerobactin* (*iucD*), P fimbriae (*pap*), and S/F1C fimbriae (*sfa/focDE*). The antibiotic-resistance phenotypes were also determined.

Results: The isolates from UTI cases were distributed within phylogroups A (31%), B_1 (10%), B_2 (28%), and D (31%). The prevalence of *iucD, hly*, *pap*, and *sfa/focDE* virulence genes was significantly associated with groups B_2 and D. The most-resisted antibiotics were cefazolin (93%) and co-trimoxazole (68%), while the isolates were most sensitive to nitrofurantoin (1%) and imipenem (2%).

Conclusions: The phylogroups of *E. coli* isolates from UTI cases showed that groups D, B_2, and A are prevalent in women in Sabzevar, as the dominant pathogenic phylogroups. The comparison showed that there was no significant difference in the occurrence of virulence factors or in the distribution of antibiotic resistance between urinary *E. coli* isolates, but the virulence genes were distributed more into groups B_2 and D, respectively. Our study showed that the highest sensitivity was to nitrofurantoin and imipenem, but the decision on a treatment strategy remains based on the physician's diagnosis and the antimicrobial-resistance tests.

Keywords: Antibiotic Resistance, Female, Urinary Tract Infections, Virulence Factors, *Escherichia coli*

1. Background

Escherichia coli is a particularly complex species that is grouped into pathotypes of partly zoonotic intestinal pathogenic *E. coli* and extraintestinal pathogenic *E. coli* (ExPEC) (1, 2). ExPEC strains can cause serious infections of certain organs and systems, including the urinary, central nervous, and blood circulatory systems and the reproductive tract (3-5).

Every year, 130 - 175 million cases of uncomplicated urinary tract infections (UTI) occur globally, and > 80% are associated with *E. coli*. UTIs alone are responsible for an estimated $1 - $2 billion annually in direct healthcare costs in some regions, such as the United States. Antimicrobial drug resistance is further adding to the cost of treating these infections because they often require more complicated treatment regimens, and result in more treatment failures (5-7).

Uropathogenic *E. coli* (UPEC), as a subgroup of ExPEC, has multiple virulence factors (VFs) that confer the potential for pathogenicity, invasion, and colonization of urinary tract sites. According to the hypothesis, UPEC strains, compared to non-pathogenic strains, acquire certain virulence factors, such as P fimbriae, S fimbriae, and afimbrial adhesin-encoding genes (including *papEF*, *sfa/focDE*, afaIBC, hemolysin [*hly*], and *aerobactin* [*iucD*]) through pathogenicity islands (8, 9).

An important step in the onset and expansion of UTI is adhesion of *E. coli* to uroepithelial cells by P fimbriae. S fimbriae has also been shown to attach efficiently to the

epithelial and endothelial cells of the lower human urinary tract. Studies have shown that *papEF* and *sfa/focDE* are essential for cystitis and/or pyelonephritis (10-12). Hemolysin, encoded by the *hly* gene, stimulates sloughing of the uroepithelial cells and bladder hemorrhage. The *aerobactin* system, which is encoded by a five-gene operon (*iucA, iucB, iucC, iucD*, and *iutA*), is an expression of the iron-acquisition system, with an important role in utilizing siderophores for scavenging iron from the environment, giving UPEC the ability to colonize and persist in iron-poor niches in the host, such as the urinary tract (13, 14).

E. coli strains, according to the presence of *chuA, yjaA*, and *TspE4.C2*, are phylogenetically divided into seven groups and subgroups as follows: subgroup A0 (group A), lacking *chuA, yjaA*, and *TspE4.C2*; subgroup A1 (group A), lacking *chuA* and *TspE4.C2* and having *yjaA*; subgroup B_{22} (group B_2), having *chuA* and *yjaA* and lacking *TspE4.C2*; subgroup B_{23} (group B_2), having *chuA, yjaA,* and *TspE4.C2*; subgroup D1 (group D), having *chuA* and lacking *yjaA* and *TspE4.C2*; and subgroup D2 (group D), having *chuA* and *TspE4.C2* and lacking *yjaA* (15, 16).

During the last decade, the European antimicrobial resistance surveillance network has reported a steady increase in the rates of invasive *E. coli* isolates resistant to common and choice antibiotics, and some strains have expressed multidrug-resistant phenotypes, leaving only limited therapeutic options (17).

2. Objectives

In the present study, the correlations between the antibiotic-resistance pattern, the presence of virulence factor genes, and the phylogroup of the *E. coli* strains from female UTI cases in Sabzevar, Iran, were evaluated, as phylogenetic typing has proved useful in predicting the pathogenic potential of extraintestinal *E. coli* (18). It has been demonstrated that distribution of *E. coli* phylogroups and their antimicrobial-resistance patterns in hosts are different in various regions due to the hosts' physiological status and the environment (15).

3. Materials and Methods

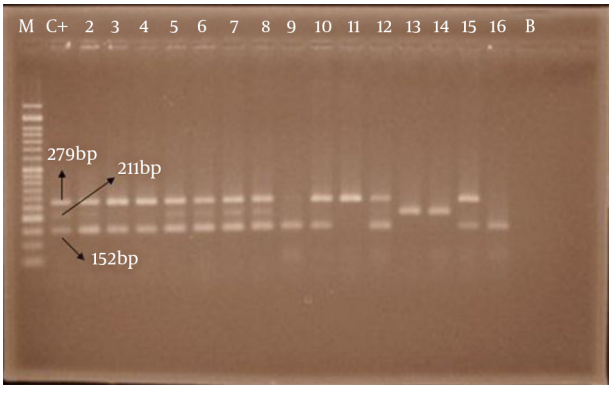
3.1. Sample Collection

One hundred fifty urine samples were collected from female patients (25 - 45 years old), suspected to have UTIs based on a urologist's diagnosis and who were referred to hospitals and independent diagnostic laboratories located in different regions of Sabzevar. The samples were cultured, and 93 *E. coli* strains were isolated from the UTIs according to the protocol described by Alonso et al. (19). The samples were directly streaked on MacConkey and EMB agar (HiMedia TM, India) for isolation of *E. coli*, then the lac+ colonies from the MacConkey agar and the lac+ colonies with metallic features from the EMB agar were selected for the biochemical identification procedure, which included indole (I) production, methyl red (MR), Voges-Proskauer (VP), and citrate utilization (C) tests. The colonies with indole$^+$, MR$^+$, VP$^-$, and citrate- characteristics were biochemically diagnosed as *E. coli*, then stock cultures were prepared from the *E. coli* isolates and stored in Luria-Bertani broth with 15% (v/v) glycerol at -20°C until genotyping.

3.2. DNA Extraction and Phylogenetic Grouping

Genomic DNA was extracted from isolated strains with the rapid one-step extraction (ROSE) method (20), and isolates were assigned to one of the four major *E. coli* phylogenetic groups (A, B_1, B_2, and D) using an established triplex PCR-based assay for the chuA, yjaA, and *tspE4.C2* genes according to the protocol of Gordon et al. (16). Briefly, the amplifications were modified and carried out in a total volume of 25 µl. Each reaction mixture contained 11.25 µl of distilled H2O, 2.5 µl of 10× buffer (supplied with Taq polymerase), 0.75 µl of MgCl2, 1 µl of dNTPs (each deoxynucleoside triphosphate at a concentration of 200 mM), 1 µl of each primer (20 pmol), 2.5 U of Taq polymerase (all from CinnaGen Co., Iran), and 3 µl of DNA template. Thermal cycler (BIO-RAD-USA) conditions were as follows: 4 minutes of initial denaturation at 94°C, followed by 30 cycles of 5-sec denaturation at 94°C, 10 seconds of annealing at 57°C, and a final extension step of 5 minutes at 72°C. A negative control (a reaction lacking the template DNA) was included in all amplifications performed, and strain ECOR62 was used as a positive control (Figure 1).

Figure 1. Positive Triplex PCR Results for the Detection of *E. coli* Phylogenetic Groups/Subgroups



M, marker (50 bp); C+, positive control for three genes (EECOR62); 2 - 16, *E. coli* isolates harboring different phylogenetic markers (test strains); B, blank as negative control (reactions lacking DNA template).

3.3. Virulence Genotyping

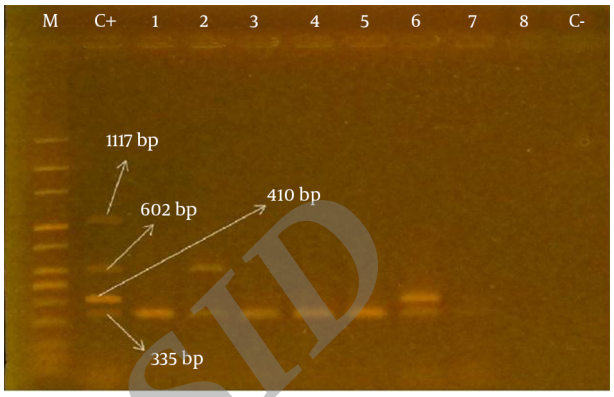
For the next step, the detection of virulence factor genes (*hly, iucD, sfa/focDE*, and *pap*) was performed according to the protocol of Adib et al. (10). The amplifications were

modified and carried out in a total volume of 25 µl. Each reaction mixture contained 8.25 µl of distilled H2O, 2.5 µl of 10× buffer (supplied with Taq polymerase), 0.75 µl of MgCl2, 1 µl of dNTPs (each deoxynucleoside triphosphate at a concentration of 200 mM), 1 µl of each primer (20 pmol), 2.5 U of Taq polymerase (all from CinnaGen Co., Iran), and 4.5 µl of DNA template. Thermal cycler conditions were as follows: 3 minutes of initial denaturation at 95°C, followed by 35 cycles of 60 seconds denaturation at 95°C, 60 seconds of annealing at 53°C, 60 seconds of extension at 72°C, and a final extension step of 10 minutes at 72°C. A negative control (reactions lacking the template DNA) was included in all amplifications performed, and the reference strains 28C (*hly*+), J96 (*sfa/focDE*+, *papEF*+), and A30 (*iucD*+) were used as positive controls (Figure 2). The primers used in this study and their sequences are submitted in Table 1.

3.4. Antimicrobial Susceptibility Testing

Disc-diffusion susceptibility testing for nine antimicrobial agents was performed on all *E. coli* isolates using the clinical and laboratory standards institute (CLSI) guidelines (CLSI, 2008a, b). The susceptibility was interpreted according to these guidelines for amikacin (AK), imipenem (IMI), gentamycin (GM), cefazolin (CS), cefepime (CPM), ciprofloxacin (CIP), co-trimoxazole (ST), nalidixic acid (NA), and nitrofurantoin (NI) (21, 22).

Figure 2. Positive PCR Multiplex Results for the Detection of *E. coli* Virulence Genes



M, mid range marker (100 bp-3 kbp); C-, negative control (blank); C+, positive control (J96, A30, and 28C strains) harboring *papEF* (335 bp), *sfa/focDE* (410 bp), *iucD* (602 bp), *hly* (1117 bp); 1 - 8, sample strains.

Table 1. The Sequences of Primers Used in This Study for Assigning *E. coli* Isolates in Phylogenetic Groups, and the Virulence Gene Distributions Between Them

	Sequence of Primers (5 - 3)	Product Size (bp)	Control (+) Strains
Phylogenetic grouping primers (16)			
chuA1	GACGAACCAACGGTCAGGAT	279	ECOR62
chuA2	TGCCGCCAGTACCAAAGACA		
yjaA1	TGCCGCCAGTACCAAAGACA	211	ECOR62
yjaA2	ATGGAGAATGCGTTCCTCAAC		
TspE4C2.1	GAGTAAGTTCGGGGCATTCA	152	ECOR62
TspE4C2.2	CGCGCCAACAAAGTATTACG		
Virulence factor detection (10)			
Sfa/focDE, 1s	CTCCGCAGAACTGGGTGCATCTTA	410	J96 (*sfa/focDE +; papEF +*)
Sfa/focDE, 2s	CGCAGGAGTAATTACAAACCTGGCA		
papEF3, 599	GCAACAGCAACGCTGGTTGCATCA	336	J96 (*sfa/focDE +; papEF +*)
papEF4, 600	AGAGAGAGCCACTCTTATACGGACA		
iucD1	TACCGGATTGTCATATGCAGACCGT	602	A30 (*iucD +*)
iucD2	AATATCTTCCTCCAGTCCGGAGAAG		
hly1	AACAAGGATAAGCACTGTTCTGGCT	1177	28C (*hly +*)
hly2	ACCATATAAGCGGTCATTCCCGTCA		

4. Results

In this study, 93 strains of *E. coli* from 150 women (62%) who had been referred to hospitals and diagnostic laboratories in Sabzevar with clinical signs of UTI, were isolated. The phylotyping of the *E. coli* strains showed 29 isolates (31%) in phylogroup A, 29 isolates (31%) in phylogroup D, 26 isolates (28%) in phylogroup B_2, and nine isolates (10%) in phylogroup B_1.

The detection of virulence genes (*hly, iucD, pap*, and *sfa/focDE*) in 93 *E. coli* isolates from UTI cases showed that the *hly* gene was present in nine (10%) isolates, including two strains (22%) belonging to phylogroup A, two strains (22%) belonging to phylogroup B_2, and five strains (55%) belonging to phylogroup D. The *iucD* gene was present in 51 (55%) isolates, including 12 strains (24%) belonging to phylogroup A, two strains (4%) belonging to phylogroup B_1, 23 strains (47%) belonging to phylogroup B_2, and 12 strains (24%) belonging to phylogroup D. The *pap* gene was present in 10 (10%) isolates, including two strains (20%) belonging to phylogroup A, four strains (40%) belonging to phylogroup B_2, and four strains (40%) belonging to phylogroup D. The *sfa/focDE* gene was present in 19 (20%) isolates, with four strains (21%) belonging to phylogroup A, 10 strains (52%) belonging to phylogroup B_2, and five strains (26%) belonging to phylogroup D (Table 2).

According to the antibiogram results, 89% of the 93 *E. coli* isolates from UTI cases were resistant to cefazolin (32% belonging to phylogroup A, 9% to phylogroup B_1, 29% to phylogroup B_2, and 30% phylogroup D), 78% were resistant to co-trimoxazole (30% belonging to phylogroup A, 13% to phylogroup B_1, 25% to phylogroup B_2, and 31% to phylogroup D), 57% were resistant to nalidixic acid (26% belonging to phylogroup A, 16% to phylogroup B_1, 34% to phylogroup B_2, and 22% to phylogroup D), 51% were resistant to amikacin (23% belonging to phylogroup A, 8% to phylogroup B_1, 31% to phylogroup B_2, and 37% to phylogroup D), 32% were resistant to gentamycin (25% belonging to phylogroup A, 12% to phylogroup B_1, 31% to phylogroup B_2, and 31% to phylogroup D), 30% were resistant to ciprofloxacin (35% belonging to phylogroup A, 21% to phylogroup B_1, 26% to phylogroup B_2, and 17% to phylogroup D), 21% were resistant to cefepime (27% belonging to phylogroup A, 5% to phylogroup B_1, 33% to phylogroup B_2, and 33% to phylogroup D), 3% were resistant to nitrofurantoin (the only strain of which belonged to phylogroup A), and 3% were resistant to imipenem (for which both resistant strains belonged to phylogroup A). Cefazolin and co-trimoxazole (11 isolates) showed the most prevalent antibiotic-resistance patterns among the strains, respectively (Table 3).

Table 2. Frequency of Virulence Genes (VG) in Relation to Phylogenetic Group Among Urinary *E. coli* Strains Isolated From UTI Samples Collected in Sabzevar, Iran[a]

FG	No. Strains	Virulence Genes (% of VG in Each FG)			
		iucD	*hly*	*pap*	*Sfa/focDE*
Total	93	51	9	10	19
A[b]	29 (31)	12 (24)	2 (22)	2 (20)	4 (21)
B_1[b]	9 (10)	2 (4)	0	0	0
B_2[b]	26 (28)	23 (47)	2 (22)	4 (40)	10 (52)
D[b]	29 (31)	12 (24)	5 (55)	4 (40)	5 (26)

[a]Source: *E. coli* strains from UTI cases.
[b]Values are expressed as No.(%)

Table 3. Frequency of Antibiotic Resistance (%) in Relation to Phylogenetic Groups Among Urinary *E. coli* Isolated From UTI Samples Collected in Sabzevar, Iran[a]

FG	No. of Strains	Antibiotic Resistance (%Resistant in Each FG)								
		AK	IMI	GM	CIP	CS	CPM	ST	NA	NI
Total	93	38	2	16	23	87	18	63	49	1
A[b]	29 (31)	9 (23)	2 (100)	4 (25)	8 (35)	28 (32)	5 (27)	19 (30)	13 (26)	1 (100)
B_1[b]	9 (10)	3 (8)	0	2 (12)	5 (21)	8 (9)	1 (5)	8 (13)	8 (16)	0
B_2[b]	26 (28)	12 (31)	0	5 (31)	6 (26)	25 (29)	6 (33)	16 (25)	17 (34)	0
D[b]	29 (31)	14 (37)	0	5 (31)	4 (17)	26 (30)	6 (33)	20 (31)	11 (22)	0

Abbrevations: AK, amikacin; CIP, ciprofloxacin; CPM, cefepime; CS, cefazolin; GM, gentamycin; IMI, imipenem; NA, nalidixic acid; NI, nitrofurantoin; ST, co-trimoxazole.
[a]Source: *E. coli* strains from UTI cases.
[b]Values are expressed as No.(%)

5. Discussion

This study was conducted to determine the distribution of phylogroups in *E. coli* strains from female UTI cases in Sabzevar, the prevalence of certain virulence genes, and the antimicrobial-resistance genes in the related isolates.

E. coli strains can be assigned to one of four main phylogenetic groups: A, B_1, B_2, or D. These phylogroups apparently differ in certain characteristics, such as virulence factors, antibiotic-resistance profiles, and ecological niches (23-26). It has been demonstrated that ExPEC strains usually belong to groups B_2 and D, and that the intestinal pathogenic strains belong to groups A, B_1, and D, while the commensal strains belong to groups A and B_1 (18, 27-29). In the present study, after analyzing 93 strains of *E. coli* from UTI cases as a subgroup of ExPEC, we found that 60% of the isolates belonged to groups B_2 and D. This was in agreement with the results of Johnson et al. and Picard et al. (18, 26, 27), who concluded that extraintestinal pathogenic *E. coli* strains usually belong to groups B_2 and D, with more virulence properties compared to other phylogroups (18, 26). Approximately 40% of the *E. coli* isolates from our study belonged to groups A and B_1, in accordance with the studies of Bingen et al. and Pupo et al., demonstrating that the route of infection can be intestinal pathogenic *E. coli* and commensal strains (28, 29), and that one of the most important causes of infection with commensal agents is poor observation of preventative criteria. It has also been demonstrated that the virulence factor properties of *E. coli* strains, the antibiotic pressure in each geographic region, and ecological differences can determine the phylogroup of isolates from UTIs in each region (30).

Extraintestinal pathogenic *E. coli* strains have multiple virulence factors that confer the potential for pathogenicity. Recently, extended virulence genotypes have been reported for ExPEC isolates, and similarities in virulence factors, phylogenetic backgrounds, and genetic profiles have been noted among *E. coli* strains from animals and humans (8, 31). It has been stated that ExPEC strains possessing virulence factors for the invasion and colonization of extraintestinal sites typically belong to phylogenetic groups B_2 and D, while commensal *E. coli* strains usually derive from groups A and B_1, lacking the specialized virulence factor genes associated with the B_2 and D strains (1, 5).

In the present study, we monitored the distribution of certain virulence factor genes (*iucD, hly, pap*, and *sfa/focDE*) in *E. coli* isolates from female UTI cases, and found that the *iucD* gene was present in 55% of the isolates, *hly* in 10%, *pap* in 10%, and *sfa/focDE* in 20%.

When compared to commensal strains, *aerobactin* biosynthetic genes are more frequently detected in *E. coli* pathogenic strains, and their incidence correlates with highly pathogenic strains (32, 33). We found that 55% of our isolates from female UTI cases harbored the *iucD* gene as a mechanism of iron uptake from the environment. Iron is an essential element for survival of *E. coli*. It facilitates numerous cellular activities, such as peroxide reduction, electron transport, and nucleotide biosynthesis (34, 35). As iron exists at low concentrations in extraintestinal sites of infection, the ExPEC strains have evolved multiple strategies for sequestering iron from the host (32). This explains why the other strains lack the *iucD* gene, as they probably have other strategies for iron uptake from the host organs. Comparing the prevalence of the *iucD* gene in our diagnosed phylogroups, we found that 72% of the *iucD* genes were present in the B_2 and D groups as the main pathogenic phylogroups of *E. coli* in the extraintestinal pathogenic strains (24% in group A and 4% in group B_1). These findings are in agreement with and confirm the previous studies (15, 33).

As indicated above, the *hly* gene was detected in 10% (9 isolates) of *E. coli* strains from female UTI cases in Sabzevar, 55% of which were distributed in phylogroup D, 22% in group A, and 22% in group B_2. The hemolysin enzyme, encoded by the *hly* gene, is secreted by uropathogenic *E. coli* strains. This enzyme causes tissue damage, facilitates bacterial distribution, and participates in bacterial pathogenesis (36, 37). It is believed that *hly* is more common among invasive uropathogenic strains than in nonpathogenic or commensal isolates of *E. coli* (38). In the present study, 77% of the *hly* genes were detected in phylogroups B_2 and D. This confirms the results of previous research showing that these phylogroups are more pathogenic and have more virulence factors than others, although different studies have shown the prevalence of *hly* in UPEC isolates to range from 2% to 47% in different countries (10, 39, 40).

UPEC isolates, like enteric *E. coli* pathogens, are a genetically heterogeneous group and can vary significantly in their ability to colonize and persist within either the bladder or the kidneys (41). Bacteria assemble adhesins on their surface as monomers, simple oligomers, or components of supramolecular fibers, called fimbriae or pili. The adhesive organelles most commonly associated with UPEC include type 1, P, and S/F1C-related pili and the Dr family of adhesins (42, 43). The *pap* and *sfa/focDE* genes monitored in our study are responsible for encoding the P pili and S/F1C pili, as important adhesins of UPEC in humans (42). We showed that the prevalence in our isolates of the *pap* gene was 10% (10 isolates) and of the *sfa/focDE* gene was 20% (19 isolates). After analyzing the relationships of these adhesion genes with our diagnosed phylogroups, it was revealed that 80% of the *pap* genes and 76% of the *sfa/focDE* genes were distributed in phylogroups B_2 and D, confirming the members of these groups to be more pathogenic strains. It seems that the other *E. coli* isolates in our study harbored genes for adhesins, such as type 1 fimbriae and members of the Dr adhesins, which can be found in some UPEC strains as the organelle used to attach and colonize in the host urinary tract.

Understanding the impact of drug susceptibility patterns is crucial, as the changing rate of antibiotic susceptibility has a large impact on the treatment of UTIs.

Treatment failure, prolonged or repeated hospitalizations, increased costs of care, and increased mortality are some of the consequences of bacterial resistance in UTIs. Although several factors may play roles in antimicrobial resistance, the misuse of antibiotics in each geographic location directly affects the antibiotic-resistance pattern (44). Antibiotic-resistant *E. coli* strains pose a therapeutic challenge, leaving few suitable alternatives with unknown intrinsic virulence potential. Their multiple virulence factors may mediate colonization of host surfaces, injury to host tissues, and avoidance or subversion of host defense systems (45-47). In our study, we investigated the antimicrobial susceptibility pattern of UPEC strains isolated from UTI cases in our region, for better usage of empiric antimicrobial agents and to demonstrate the distribution of resistance patterns in the related phylogroups of *E. coli* bacteria. We monitored the antimicrobial resistance of 93 *E. coli* isolates from UTI cases in the Sabzevar region with the disk diffusion method. Nine antimicrobial agents, as described above, were applied for assessment of resistance patterns. These patterns showed that the most phenotypic resistance in our isolates was for cefazolin (93%), followed by co-trimoxazole (68%), nalidixic acid (52%), amikacin (40%), ciprofloxacin (25%), cefepime (19%), gentamycin (17%), imipenem (2%), and nitrofurantoin (1%), respectively. There was no significant relationship between specific phylogroups and antimicrobial-resistance patterns in the tested isolates. Characterization of antibiotic-resistant *E. coli* strains from UTIs in human cases resident in our region revealed a wide range of phenotypic resistance patterns between our isolates, and the most resistant strains were genotypically diverse across the phylogroups.

Several investigations have been conducted to determine the antibiotic-resistance patterns of UPEC strains from different regions of Iran, and different results have been obtained, demonstrating the diverse resistance patterns between isolates (48-52). In the present study, the antibiotics nitrofurantoin (with 1% resistance in all isolates) and imipenem (with 2% resistance) seem to be the best antimicrobials used in the region.

Monitoring of the emergence and spread of dominant antimicrobial-resistant strains within hospital surveillance programs may assist urologists in developing improved strategies for the treatment and prevention of infections for which the choice of antimicrobials is limited. The authors recommend that urologists review the antibiotic-resistance patterns in their region. The antibiogram test, the availability of antimicrobials for each UTI case, and the physician's decision based on clinical signs and limitations remain, of course, the most important guidelines for the initiation of an antimicrobial treatment strategy.

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Footnote

Authors' Contribution: Hamid Staji: study design, preparing the manuscript, and data analysis; Javad khoshgoftar: laboratory practice; Abbas Javaheri Vayeghan: preparing the manuscript; Mohammad Reza Salimi Bejestani: English editing of the manuscript.

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