Original Article

Distribution of Virulence Factors According to Antibiotic Susceptibility among *Escherichia coli* Isolated from Urinary Tract Infection

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Abstract

Escherichia coli is the major causative pathogen of urinary tract infection (UTI) in humans. Virulence and drug resistance play important roles in the pathogenesis of *E. coli* infections. The aims were to investigate the presence of uropathogenic virulence genes and to evaluate a relationship between antibiotic resistance and virulence in *E. coli* from UTI. A total of 132 *E. coli* were collected between April and June 2015 in two hospitals of Sanandaj, Iran. Isolates were examined for susceptibility to 16 antibiotic disks using the disk diffusion method and for possession of virulence genes by polymerase chain reaction. Associations between antimicrobial resistance and virulence genes were investigated. A $P < 0.05$ was considered significant. Of the 132 isolates, the most prevalent virulence gene was *pap* (31.1%), followed by *cnf* (28.8%), *hly* (16.7%), and *afa* (10.6%). Different patterns of virulence genes were identified. A significant association was detected between the simultaneous presence of *hly* and *pap*. The most effective antibiotics were nitrofurantoin, cefoxitin, and imipenem and the least effective were ampicillin, trimethoprim-sulfamethoxazole, and cefotaxime. An association was seen between the presence of *cnf* and susceptibility to the certain antibiotics, whereas strains with a reduced susceptibility to the certain antibiotics were associated with a significantly increased prevalence of *afa* and *hly* ($P < 0.05$). These findings suggest a correlation between the presence of virulence gene and resistance in *E. coli* strains from UTI. The results indicate that there is a need for surveillance programs to monitor drug resistance in pathogenic *E. coli*.

Keyword: *Antimicrobial agents, Escherichia coli, resistance, urinary tract infection, virulence*

Introduction

Pathogenic strains of *Escherichia coli* have the potential to cause a wide variety of infectious diseases, including neonatal meningitis, septicemia, intestinal tract infection, and urinary tract infections (UTIs).[1] UTI is one of the most frequent bacterial infections, affecting both inpatients and outpatients, and *E. coli* is the major causative pathogen.[2] Due to anatomical differences and the hormonal milieu of the urinary tract, the probability of developing a UTI in women is significantly more than men. It is estimated that around 50%–60% of women will experience a UTI during their lifetime.[3]

UTI caused by *E. coli* strains remains an important health problem in many countries and leads to considerable morbidity costs.[1,4,5] These strains encode various virulence factors such as toxins, capsules, invasins, and adhesins, which contribute to enhanced pathogenicity. The severity of UTI reflects the virulence profile of the infecting strain, and the frequency of expression of virulence factors is higher in more pathogenic strains.[6]

Pyelonephritis-associated pilus (Pap), afimbrial adehsin (Afa), α-hemolysin (HlyA), and cytotoxic necrotizing factor 1 (CNF1) are among the most important virulence factors of *E. coli* involved in the development of UTI.[7-9] Pap is one of the most commonly found adhesins. Binding of P-fimbrial adhesin to the cell receptors of renal tissue leads to mucosal inflammation and tissue damage.[7] Afa has been implicated in the occurrence of recurrent and chronic UTIs.[10] Apart from adhesins, exotoxins such as HlyA and CNF1 are also implicated in the pathogenesis of UTIs. HlyA encoded by *hly* is the most important secreted virulence factor of *E. coli* isolated from UTI. This toxin is able to lyse host cells for crossing of the mucosal barriers, having access to host nutrients and iron stores, and damaging effector immune cells such as T-lymphocytes and neutrophils. CNF1 has been shown to have a role in

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dissemination of strains in the urinary tract. This toxin causes bladder cell exfoliation and increases bacterial access to the underlying tissue.[8]

Another problem is antibiotic resistance of the *E. coli* strains. The emergence of resistance limits the utility of older agents, such as trimethoprim-sulfamethoxazole (SXT), and increases reliance on newer broad-spectrum agents, such as extended-spectrum cephalosporins and fluoroquinolones. Unfortunately, emerging resistance now threatens the use of these newer agents as well.[11-13] Resistance to antimicrobial agents is often associated with the spread of transmissible plasmids, which may also carry virulence determinants.[14] Some studies showed that among clinical isolates of *E. coli*, the production of virulence factors is negatively associated with resistance to some antibiotics.[15,16] On the other hand, such phenomenon was not observed in other studies.[17-19] The acquisition of resistance and virulent traits may provide a benefit for the survival of microorganism. This situation may lead to ecological changes and domination of virulent antibiotic-resistant bacteria in the environment.[14]

Hence, the aims of this study were to determine the presence of the most important virulence genes involved in the development of UTI including *pap*, *afa*, *cnf-1*, and *hly*[7-9] among *E. coli* isolated from UTI and to evaluate a possible association between virulence factors and susceptibility to antimicrobial agents.

Materials and Methods

Bacterial isolates and identification

In this cross-sectional study, from April to June 2015, 132 consecutive nonduplicate *E. coli* were isolated from patients with UTI admitted to Besat and Tohid Tertiary Hospitals in Sanandaj, Iran. Sanandaj is the center of Kurdistan Province in the west of Iran, with a population of more than 300,000. The Besat and Tohid Hospitals are referral and teaching hospitals, which are affiliated to Kurdistan University of Medical Sciences. UTI was defined according to the 2015 European Association of Urology guidelines.[20] *E. coli* isolates were identified according to the standard bacteriological and biochemical tests[21] including Gram staining, fermentation of lactose, motility, ability to produce indole, and lysine decarboxylation. All bacterial isolates were preserved at −70°C in Trypticase soy broth (Quelab, New Mexico, USA), containing 15% v/v glycerol for further investigations.

Antimicrobial susceptibility testing

Susceptibility of *E. coli* isolates was determined to 16 antibiotics using the disk diffusion method on Mueller-Hinton agar plates (Merck, Germany) as recommended by the 2014 Clinical and Laboratory Standards Institute guidelines.[22] The following antibiotic disks (Rosco company, Denmark) were tested: ampicillin (AM) (10 μg), cefotaxime (CTX) (30 μg), ceftazidime (CAZ) (30 μg), imipenem (IPM) (10 μg), amoxicillin/clavulanic acid (AMC) (20/10 μg), aztreonam (AZT) (30 μg), ciprofloxacin (CP) (5 μg), tetracycline (TE) (30 μg), SXT, gentamicin (GM) (10 μg), cefepime (FEP) (30 μg), cefoxitin (CFO) (30 μg), amikacin (AN) (30 μg), norfloxacin (NOR) (10 μg), nalidixic acid (NA) (30 μg), and nitrofurantoin (FM) (300 μg). *E. coli* ATCC 25922 was used as a quality control strain.

DNA extraction and detection of virulence factors by polymerase chain reaction

Genomic DNA was extracted from *E. coli* strains by the freeze-thaw method and used as the template for polymerase chain reaction (PCR) reactions.[23] For DNA extraction, bacterial pellets prepared from 1.5 ml of an overnight culture in brain–heart infusion broth (Quelab, New Mexico, USA) were suspended in 200 μl sterile distilled water, and the suspensions were heated at 100°C for 10 min. The suspensions were then immediately placed on ice for 5 min. Samples taken through a total of three cycles of freezing-thawing were centrifuged, and the supernatants were stored at −20°C as DNA template stocks.

Detection of virulence genes was carried out by PCR. Amplification of *pap*, *afa*, *cnf-1*, and *hly* was performed using published primer pairs (SinaClon, Iran) as follows: 5′-AACAAGGATAAGCACTGTTCTGGCT-3′, 5′-ACCA TATAAGCGGTCATTCCCGTCA-3′ (for *hly*, amplicon size: 1177 bp);[24] 5′-AAGATGGAGTTTCCTATGCAGGAG-3′, 5′-CATTCAGAGTCCTGCCCTCATTATT-3′ (for *cnf*, amplicon size: 498 bp);[24] 5′-GCTG GGCAGCAAACTGATAACTCTC-3′, 5′-CATCAAGCTGTTTGTTCGTCCGCCG-3′ (for *afa*, amplicon size: 750 bp);[25] and 5′-GA CGGCTGTACTGCAGGGTGTGGCG-3′, 5′-ATATCCTTTCTGCAGGGATGCAATA-3′ (for *pap*, amplicon size: 328 bp).[25]

Amplification reactions were done in a total volume of 25 μl containing 3 μl DNA extract, 1 U of Taq DNA polymerase, 0.4 μM of each primer, 1X reaction buffer, 1.5 mM $MgCl_2$, and 200 μM of each dNTP (SinaClon, Iran). The PCR was performed in a thermal cycler (Eppendorf, Germany) under the following conditions: initial denaturation of 5 min at 94°C followed by 35 cycles of denaturation of 1 min at 94°C, annealing of 1 min at 65°C, extension of 1 min at 72°C, and a final extension of 7 min at 72°C. Conditions were the same for all genes.

The amplified products were separated on a 1% agarose gel (SinaClon) in 0.5X tris-borate EDTA buffer alongside an appropriate molecular size marker (100 bp Plus DNA ladder, SinaClon). The amplified products were visualized after staining with DNA safe stain (SinaClon) and photographed using a UV transillumination imaging system. Positive controls were kindly given by

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Statistical analyses

Data were analyzed using SPSS software version 16.0 (SPSS, Chicago, IL, USA). The relationship between virulence factors and antibiotic susceptibility was determined using Pearson's Chi-square test or Fisher's exact test. To facilitate our analysis, the isolates showing intermediate susceptibility were grouped with the resistant strains. A $P < 0.05$ was considered significant.

Results

A total of 132 *E. coli* strains were collected from patients with UTI between April and June 2015. The average age of the patients was 35.7 years; the oldest patient was 93 years (one patient) and four 1-year-old children were the youngest patients. Among the 132 isolates, 107 isolates (81.1%) were from females and 25 (18.9%) were from males. Forty-seven (35.6%) of the 132 isolates were from hospitalized patients and 85 (64.4%) were from outpatients.

Antimicrobial susceptibility

Antimicrobial susceptibility results showed that all isolates (97%) were susceptible to FM, except for four isolates. Of the 132 isolates, 122 (92.4%) were susceptible to CFO, 116 (87.9%) to IPM, 99 (75%) to AN, and 95 (72%) to AMC. The susceptibility rate of isolates to 16 antimicrobial agents is presented in Table 1. Compared with the isolates from inpatients, except for AMC, FM, IPM, AN, and GM, the frequency of susceptibility to the antimicrobial agents was higher or similar in the outpatients isolates [Table 1].

Distribution of virulence genes

Prevalence of virulence genes was analyzed by PCR. Of the 132 isolates, the *pap* gene was found in 41 (31.1%) isolates, the *cnf* in 38 (28.8%), the *hly* in 22 (16.7%), and the *afa* in 14 (10.6%) isolates. Fifty-seven strains were negative for all the studied virulence genes. The prevalence of virulence genes was higher in males, except for *afa,* which was detected in a higher prevalence in females than in males (11.2% vs. 8%) [Table 2]. Among the studied virulence genes, only *pap* showed a meaningful difference of distribution according to sex group ($P = 0.01$).

Except *pap* gene, that its prevalence in the inpatient group was higher than outpatient group (16/47 [34%] vs. 25/85 [29.4%]), the prevalence of other virulence genes was higher in the outpatient group. The *hly*, *cnf*, and *afa* were detected in 7 (14.9%), 12 (25.5%), and 2 (4.3%) of the 47 strains collected from inpatients and in 15 (17.6%), 26 (30.6%), and 12 (14.1%) of the 85 strains collected from outpatients, respectively. However, the prevalence of virulence genes was not significantly different between the two groups ($P > 0.05$).

Table 1: Antimicrobial susceptibility of 132 *Escherichia coli* isolated from urinary tract infection inpatient and outpatient groups

Antibiotic	Susceptibility, *n* (%)		
	Inpatients (*n*=47)	Outpatients (*n*=85)	Total (*n*=132)
FM	46 (97.9)	82 (96.5)	128 (97)
CFO	41 (87.2)	81 (95.3)	122 (92.4)
IPM	43 (91.5)	73 (85.9)	116 (87.9)
AN	37 (78.7)	62 (72.9)	99 (75)
AMC	36 (76.6)	59 (69.4)	95 (72)
NOR	30 (63.8)	56 (65.9)	86 (65.2)
CAZ	28 (59.6)	56 (65.9)	84 (63.6)
AZT	27 (57.4)	57 (67.1)	84 (63.6)
CP	25 (53.2)	53 (62.4)	78 (59.1)
GM	26 (55.3)	45 (52.9)	71 (53.8)
NA	19 (40.4)	43 (50.6)	62 (47)
FEP	20 (42.6)	41 (48.2)	61 (46.2)
TE	16 (34)	37 (43.5)	53 (40.2)
CTX	14 (29.8)	30 (35.3)	44 (33.3)
SXT	10 (21.3)	30 (35.3)	40 (30.3)
AM	11 (23.4)	27 (31.8)	38 (28.8)

NA: Nalidixic acid, FM: Nitrofurantoin, CFO: Cefoxitin, IPM: Imipenem, AN: Amikacin, NOR: Norfloxacin, CAZ: Ceftazidime, AZT: Aztreonam, CP: Ciprofloxacin, GM: Gentamicin, FEP: Cefepime, TE: Tetracycline, CTX: Cefotaxime, SXT: Trimethoprim-sulfamethoxazole, AM: Ampicillin, AMC: Amoxicillin/clavulanic acid

Table 2: Prevalence of the virulence genes among 132 *Escherichia coli* isolates from urinary tract infection in males and females

Gene	*n* (%) of positive strains in female patients (*n*=107)	*n* (%) of positive strains in male patients (*n*=25)	*P*
hly	17 (15.9)	5 (20)	0.61
cnf	29 (27.1)	9 (36)	0.37
afa	12 (11.2)	2 (8)	0.63
pap	28 (26.2)	13 (52)	0.01*

*$P<0.05$

To identify phenotypes of the isolates, the prevalence of profiles of the virulence genes was ascertained. A total of 75 (56.8%) isolates were found to harbor at least one of the four urogenes investigated. The maximum number of detected amplicons in one strain was three of the virulence gene regions targeted. Combinations of adhesin and toxin genes encoded by *E. coli* isolates are presented in Table 3. Considering all virulence genes together, the studied strains exhibited 11 virulence gene profiles, referred to as urovirulence profile (UP) followed by an Arabic numeral. Of these 11 combinations, the most common gene profile was UP1, which was characterized by the presence of only the *cnf* gene (16 isolates) followed by UP2, which was found in 15 isolates and characterized by the presence of the *pap* gene only. The least distributed profile was UP11, which was detected in only one strain and characterized by the simultaneous presence of *hly* and *afa* genes.

A significant association was detected between the simultaneous presence of *hly* and *pap* genes ($P < 0.001$), which corresponded to 14 (10.6%) strains. Sixteen isolates carried both *pap* and *cnf*, 10 strains *hly* and *cnf*, and three isolates *pap* and *afa* but lacked significance according to the Chi-square and Fisher's tests.

Associations between antimicrobial resistance and virulence traits

Table 4 shows the prevalence of each virulence gene according to antibiotic susceptibility status of isolates. In general, *pap* was more prevalent in nonsusceptible isolates group (resistant plus intermediately resistant [for FEP: susceptible dose dependent]). Exceptions were beta-lactam antibiotics (IPM, CAZ, FEP, AZT, and CFO), for which susceptible isolates showed a higher prevalence of the *pap* gene. The *afa* gene was also more prevalent in nonsusceptible isolates, except TE, AN, FM, and AMC, for which the susceptible isolates showed a higher prevalence. By contrast, the *cnf* gene was more prevalent in susceptible isolates; the only exceptions were GM and FM for which nonsusceptible isolates showed a higher prevalence. The *hly* was almost equally distributed between susceptible and nonsusceptible groups although was slightly shifted toward susceptible isolates group and away from nonsusceptible isolates [Table 4].

Table 3: Virulence patterns identified among 132 *Escherichia coli* isolated from urinary tract infection

Profile	Virulence genes				Total number of strains
	hly	*cnf*	*afa*	*pap*	
UP1	-	+	-	-	16
UP2	-	-	-	+	15
UP3	-	+	-	+	9
UP4	-	-	+	-	7
UP5	+	-	-	+	7
UP6	+	+	-	+	7
UP7	+	-	-	-	4
UP8	-	+	+	-	3
UP9	-	-	+	+	3
UP10	+	+	-	-	3
UP11	+	-	+	-	1

UP: Urovirulence profile, +: Positive, -: Negative

The possible statistical association between antibiotic susceptibility/nonsusceptibility phenotypes and virulence genes of isolates was subsequently investigated. We found that the distribution of *hly* and *pap* in relation to antimicrobial resistance phenotypes was not different ($P > 0.05$), except that AN-susceptible isolates significantly exhibited a lower prevalence of the *hly* ($P < 0.05$). A more analysis revealed two further groups of associations: first, an association between the presence of *afa* and nonsusceptibility to beta-lactams (CTX and FEP) and the quinolone NA as well as the fluoroquinolone CP ($P < 0.05$), and second, an association between the presence of *cnf* and susceptibility to beta-lactams (AM, IPM, CAZ, FEP, AMC), quinolone (NA), and fluoroquinolones (CP, NOR) ($P < 0.05$) [Figure 1].

Table 4: Distribution of virulence genes according to antibiotic susceptibility among 132 *Escherichia coli* isolated from urinary tract infection

Antibiotics	Prevalence of *pap* (%)		Prevalence of *afa* (%)		Prevalence of *hly* (%)		Prevalence of *cnf* (%)	
	Susceptible isolates	Nonsusceptible isolates[b]	Susceptible isolates	Nonsusceptible isolates	Susceptible isolates	Nonsusceptible isolates	Susceptible isolates	Nonsusceptible isolates
CP (*n*[a]=78)	28.2	35.2	3.84	20.37	16.7	16.7	35.9	18.5
SXT (*n*=40)	25	33.7	5	13	10	19.6	37.5	25
GM (*n*=71)	29.6	32.8	8.5	13.1	15.5	18	28.2	29.5
AM (*n*=38)	23.7	34	5.3	12.8	13.2	18.1	42.1	23.4
CTX (*n*=44)	25	34.1	0	15.9	18.2	15.9	34.1	26.1
TE (*n*=53)	28.3	32.9	11.3	10.1	17	16.5	34	25.3
IPM (*n*=116)	32.8	18.8	10.3	12.5	15.5	25	31.9	6.2
NOR (*n*=86)	30.2	32.6	7	17.4	15.1	19.6	34.9	17.4
CAZ (*n*=84)	32.1	29.2	7.1	16.7	19	12.5	36.9	14.6
NA (*n*=62)	29	32.9	3.2	17.1	21	12.9	37.1	21.4
AN (*n*=99)	27.3	42.4	11.1	9.1	12.1	30.3	30.3	24.2
FM (*n*=128)	29.7	75	10.9	0	15.6	50	28.1	50
FEP (*n*=61)	32.8	29.6	4.9	15.5	23	11.3	37.7	21.1
AMC (*n*=95)	28.4	37.8	11.6	8.1	18.9	10.8	35.8	10.8
AZT (*n*=84)	32.1	29.2	7.1	16.7	19	12.5	34.5	18.8
CFO (*n*=122)	31.1	30	9.8	20	18	0	31.1	0

[a]*n*: Number of susceptible isolates to the antibiotic, [b]Nonsusceptible: resistant plus intermediately resistant [for FEP: SDD].
CP: Ciprofloxacin, SXT: Trimethoprim-sulfamethoxazole, GM: Gentamicin, AM: Ampicillin, CTX: Cefotaxime, TE: Tetracycline, IPM: Imipenem, NOR: Norfloxacin, CAZ: Ceftazidime, NA: Nalidixic acid, AN: Amikacin, FM: Nitrofurantoin, FEP: Cefepime, AMC: Amoxicillin/clavulanic acid, AZT: Aztreonam, CFO: Cefoxitin, SDD: Susceptible dose dependent

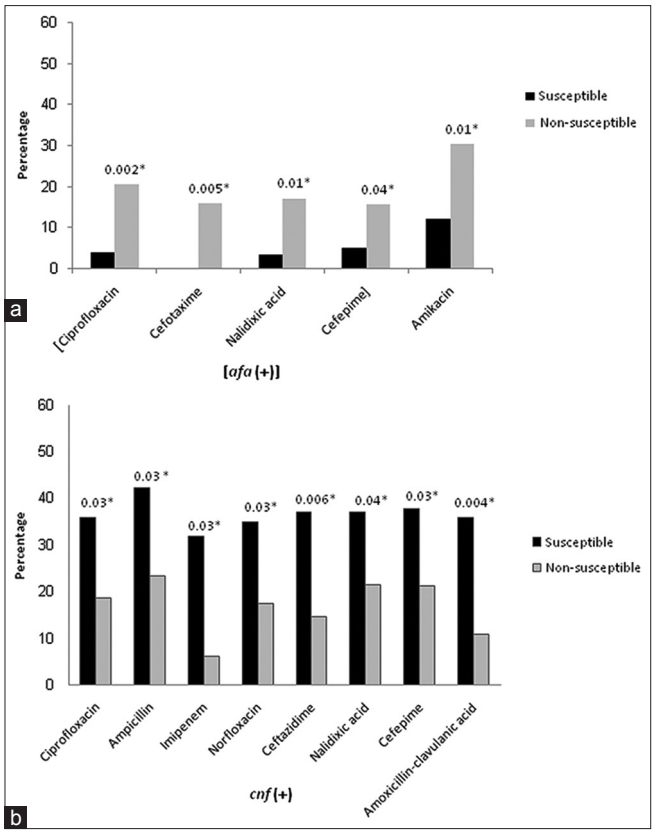

Figure 1: Relationships between virulence factors and antimicrobial susceptibility: (a) susceptibility to the antibiotics shown was significantly related to the lower prevalence of *hly* and *afa* genes. (b) A significant relationship between presence of *cnf* gene and susceptibility to antibiotics (*$P < 0.05$)

Discussion

UTI is one of the important bacterial infections, affecting both inpatients and outpatients, and *E. coli* is the leading causative agent of UTI. It is thought that the pathogenic potential of *E. coli* isolates is dependent on the presence of various virulence factors.[1] In our study, of the 132 *E. coli* isolated from UTI, the *pap* gene was present in 31.1%, the *afa* in 10.6%, the *cnf* in 28.8%, and the *hly* in 16.7% of isolates. The *hly* was found to be significantly associated with *pap.* The *pap* adhesion gene was the most commonly identified virulence gene, in agreement with other reports.[10,26,27] It has been shown that transformation of *E. coli* with *pap* sequence makes it a more potent inducer of the host response. It is conceivable that in some strains of *E. coli*, *pap* has important role in the progression to more severe infections of the urinary tract.[6] Isolates from outpatients showed a higher prevalence of the studied virulence genes except *pap* gene.

In recent years, management of UTIs has become increasingly problematic due to the emergence of drug-resistant *E. coli* strains in many countries.[11,13,28] The emergence of resistance limits the utility of older agents, such as SXT, which results in increased reliance on newer broad-spectrum agents, such as extended-spectrum cephalosporins and fluoroquinolones.[29] Antibiotic sensitivity test in our isolates showed FM as the most effective followed by CFO and IPM. AM, SXT, and CTX were the least effective antibiotics. The isolates from outpatients were more resistant to FM, IPM, AMC, AN, and GM than those from inpatients, in particular AMC. The likely reasons for the high resistance rates to these antibiotics among outpatients are the inappropriate use of these antimicrobials, ineffective infection control and health programs, and cross-resistance among antibiotics of the same class, such as AN and GM.[30] In humans, 80%–90% of antimicrobial drugs are used in outpatients, and it is estimated that 20%–50% of the use of antibiotics is questionable.[31] Resistant strains can be traced from the community to hospitals and contribute to multidrug resistance in hospital settings.[32]

Several studies indicate that resistance to some antibiotics is associated with decreased virulence traits among clinical *E. coli* isolates.[15,16] On the other hand, such phenomenon was not observed in some other studies.[17-19] We observed an association between the presence of *cnf* and susceptibility to the tested beta-lactams, quinolone, and fluoroquinolones [Figure 1]. However, *E. coli* strains with a reduced susceptibility to the studied extended-spectrum cephalosporins (CTX and FEP), quinolone NA, and fluoroquinolone CP were associated with a significantly increased prevalence of *afa*. *E. coli* strains expressing adhesins of the Afa family have unique renal tissue tropisms that favor the establishment of chronic and/or recurrent infections.[8] Moreover, isolates with a reduced susceptibility to AN significantly exhibited a higher prevalence of the *hly*, resulting in a slightly increased inferred virulence potential compared with susceptible isolates. Compared to their susceptible counterparts, resistant bacterial infections with more virulence factors are generally associated with increased morbidity, mortality, and treatment costs.[33]

The reasons for this correlation are not entirely clear. A biological basis for the association of antimicrobial resistance with virulence genes in *E. coli* has been previously reported for certain genes; for example, an 80-megadalton plasmid coding for AM resistance has been associated with genes for ST (heat-stable enterotoxin) synthesis.[34] Recently, some *in vitro* studies have shown that decreased pathogenicity of *E. coli* is associated with the acquisition of quinolone resistance.[35] Soto *et al*. suggested that subinhibitory concentrations of quinolones induce the SOS system response (DNA repairing mechanism), which could favor *in vitro* loss of virulence genes in *E. coli* strains. According to their results, the virulence genes may have been lost in exchange for resistance.[36] However, the finding that spontaneous fluoroquinolone-resistant mutants derived from hemolytic fluoroquinolone-susceptible strains are still able to produce hemolysin[18] suggested otherwise. Although one hypothesis does not exclude the other, further studies are needed to consolidate the findings. It is

also possible that ecological factors such as the geographic origin of isolates represent important additional factors that should be considered.[37] It is worthwhile to investigate whether gene linkage on plasmids or other mobile genetic elements underlies the associations observed in our study.

In conclusion, this study describes the prevalence of resistance phenotypes and virulence genes in *E. coli* isolates from UTI in Kurdistan Province, Iran. The study also shows the relationship between antimicrobial resistance and virulence genes. The increasing emergence of antimicrobial resistance and relationships between resistance and virulence genes suggest that there is a great need for surveillance programs to monitor drug resistance in pathogenic bacteria. Such surveillance programs would provide appropriate guidelines for restriction of antimicrobial use and would be important steps in efforts to understand, prevent, and control the emergence and spread of antimicrobial resistance and virulence genes.

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Conflicts of interest

There are no conflicts of interest.

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