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Identification and Evaluation of Pathogenic Genes (*traT, hly, aer, pap,* and *fimH*) and Antibiotic Resistance Genes (bla_{TEM}, bla_{SHV}, and bla_{CTX}) in *Escherichia coli* in Patients Referred to Gonabad Hospitals, Iran

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Abstract

Background: Urinary tract infection (UTI) is one of the common bacterial infections. *Escherichia coli* is the most common cause of UTI. In this research, the prevalence of several virulence factors and beta-lactam resistance genes was investigated.

Methods: One hundred *E. coli* isolates were collected from patients' specimens with UTI referred to Allame-Bohlol Gonabadi hospital. Polymerase chain reaction (PCR) was performed to identify five pathogenic genes (*fimH, aer, pap, hly, traT*) and three antibiotic resistance genes (bla_{TEM}, bla_{CTX}, bla_{SHV}).

Results: The frequencies of bla_{SHV}, bla_{TEM} and bla_{CTX} beta-lactamase genes among extended-spectrum-beta-lactamases (ESBLs) positive isolates were 11.1%, 48.1%, and 93.3%, respectively. A significant number of isolates were resistant to the most commonly used antibiotics.

Conclusion: Pathogenic genes may also increase the severity, progression, and expansion of urinary tract infections. Therefore, identifying these genes as critical controllers of illness can use for better manage the treatment.

Keywords: Antibiotic resistance genes, *E. coli*, Pathogenic genes, Polymerase chain reaction.

Introduction

Escherichia coli is both a commensal agent and a pathogen, causing extraintestinal and intestinal infections (1). It triggers a wide-range of clinical diseases in the urinary tract, including kidney infection (pyelonephritis), bladder infection (cystitis) and asymptomatic bacteriuria (ABU). Also, half of the bloodstream infections originating in the urinary tract occur due to urinary tract infections (UTIs) with *E. coli* (2-4). One of the most common hospital- and community-acquired infections caused by *E. coli* is UTIs due to high mortality (5). Uropathogenic *E. coli* (UPEC) is a strain with a significant ability to cause UTIs. UPEC has further been classified with *E.coli* strains that cause prostatitis, meningitis, and bacteremia as ExPEC (extraintestinal pathogenic *E.coli*) (6). This, which is considered to be an opportunistic pathogen, with the human and animal gut as its reservoir (7). Pathogenic factors such as adhesion, toxin, and iron absorption systems are often located on pathogenic islands, so they are easily transmitted (8). *E. coli*

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commonly acquires antimicrobial-resistance (AMR) genes from mobile-genetic-elements(MGE), such as transposons, insertion sequences, plasmids and gene cassettes/integrons (9). MGE can also encode pathogenic factors, and there may be an interaction between virulence factors(VF) and AMR genes (9). Although the virulence factors and pathogenesis of *E. coli* in UTI have been defined, the genotypic traits that recognize non-virulent *E. coli* from UPEC have not been fully defined. The identification of the determining UPEC form is hindered by factors including numerous potentially excessive bacterial virulence-associated genes (VAGs), the distinction of UPEC from commensal *E. coli* in the intestinal microbiome, and differences in host sensitivity to UTI (10). The surface virulence factors(adhesins) such as P fimbriae are encoded by *pap* genes and are the main adhesion factors (11). S fimbrial adhesion factor, encoded by *sfa* genes, represent another type of virulence factor (12). A fimbrial adhesion factor in *E. coli* is encoded by *afa* genes (13). Additionally, the main fimbrial subunit of type 1 fimbriae is encoded by *fimA* in *E. coli* (14). Toxins are another essential type of virulence factor in *E. coli*. The α-hemolysin (HlyA) pathogenic factor, cytotoxic necrotizing factor, and aerobactin are encoded by the *hly*, *cnf*1(7), and *aer* genes, respectively (15). The various pathotypes of intestinal pathogenic *E. coli* such as enteroinvasive *E. coli* (EIEC), enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC) and enterotoxigenic *E. coli* (ETEC) are differentiated genotypically by their characteristic pathogenic genes (16). These pathotypes are usually the causes of watery diarrhea and dysentery. In contrast, *E. coli* strains are often grouped presumptively as UPEC or ExPEC based on to their site of isolation and infection, irrespective of their intrinsic pathogenesis, which is usually unbeknown (16). *E. coli* is divided into several phylogenic classes such as A, B1, B2, C, D, F, and G, of which the ExPEC and UPEC strains are mainly B2 and D. These two groups usually have more virulence factors than the other groups (7, 17, 18). Phylogroups D and B2 encompass the widespread UPEC clonal complex CC95, CC73, CC131 and, CC69 which are most cases of *E. coli* pyelonephritis, bloodstream system and cystitis contamination around the world. The prevalence of extraintestinal *E. coli* contamination, suggests that particular hereditary determinants strengthen the development (worldwide spread) and exacerbation of infections of these bacteria (19).

Broad AMR may have contributed to the later dispersal of CC131 and CC69 (19-24). *E. coli* ST131 is known for its high-quality exchange capacity and can set up resistance to numerous vital antimicrobial bunches, such as fluoroquinolones and third-generation cephalosporin (14). The supremacy of ESBLs-producing bacilli has expanded drastically around the world (23), and the CTX-M-type of ESBLs is the most important visit type (20). ESBLs-producing bacilli regularly show multidrug resistance, and related qualities are encoded by plasmids exchanged from species to species. ESBLs-producing microscopic organisms have already caused nosocomial contaminations, But now they are among the causes community-acquired contaminations; moreover, reports of urinary tract (UT) diseases caused by ESBLs-producing microbes in children have increased (25). Due to different patterns of virulence factors among *E coli* strains causing UTI in other geographical locations and increased prevalence of MDR strains in different regions, the present study aimed to investigate the extent of antimicrobial resistance and identify pathogenic genes in the *E. coli* strains isolated from UTI patients in the Gonabad city.

Materials and Methods

Sample collection and isolation of bacteria

This project was approved by the Ethics Committee of Shahid Sadoughi university of medical sciences with code IR.ssu.medicine.rec.1396.215. It is implemented in Allame-Bohlol Gonabadi hospital in Gonabad in northeastern Iran. Urine specimens were collected from individuals with complaints and symptoms of urinary tract

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infection at two hospitals in Gonabad between August 2017 and February 2018. After microscopic observation, the specimens were cultured on 5% blood-agar and EMB-agar medium (Company BioMaxima, Poland). The samples were incubated for 24 hours at 37 °C in an aerobic incubator. UTI is defined as a single organism in urine at 10^3 to 10^5 colony-forming-units (CFU) per milliliter (CFU/mL). In some culture media, different colonies were considered as contaminants and were excluded from the research. To final identify the gram-negative bacteria grown on the culture medium, Gram staining was performed and to identify the gallery culture medium, SIM medium, MR-VP test, TSI-agar and Simmons' citrate-agar and urea-agar (Merk Co, Germany) was used. One hundred *E. coli* isolates from urine samples were included in the research.

Antimicrobial susceptibility testing

Antibiotic susceptibility testing (AST) was done for all isolates on Müller-Hinton-agar (Merk Co., Germany) plates by disk diffusion method for the following antibiotics: cefixime, cefazolin, ceftriaxone, ceftazidime, cefuroxime, ciprofloxacin, ofloxacin, trimethoprim, cefotaxime, sulfamethoxazole, ampicillin, Cefoxitin, nitrofurantoin, tetracycline, amoxicillin, piperacillin/tazobactam, ertapenem, imipenem, doripenem, meropenem, Colistin (Mast Co., UK). The procedure was performed according to the CLSI standard guidelines. *E. coli* ATCC 25922 standard strain was applied to control the quality of AST. Isolates insensitive to more than one antibiotic are defined as MDR.

ESBL phenotypic diagnosis

Isolates were surveyed for ESBLs production using the combined disc method using cefotaxime (30 µg) and ceftazidime (30 µg) disks in combination with clavulanic acid (10 µg) disks as described by the CLSI method (Figure 1). Based on the CLSI guidelines, an increase of 5 mm in diameter in the inhibitory areas around the hybrid disk compared to the inhibitory areas around individual antibiotics. *E. coli* strain ATCC 25922 and *Klebsiella pneumonia* ATCC 700603 were used as negative and positive control strains of the procedure, respectively.

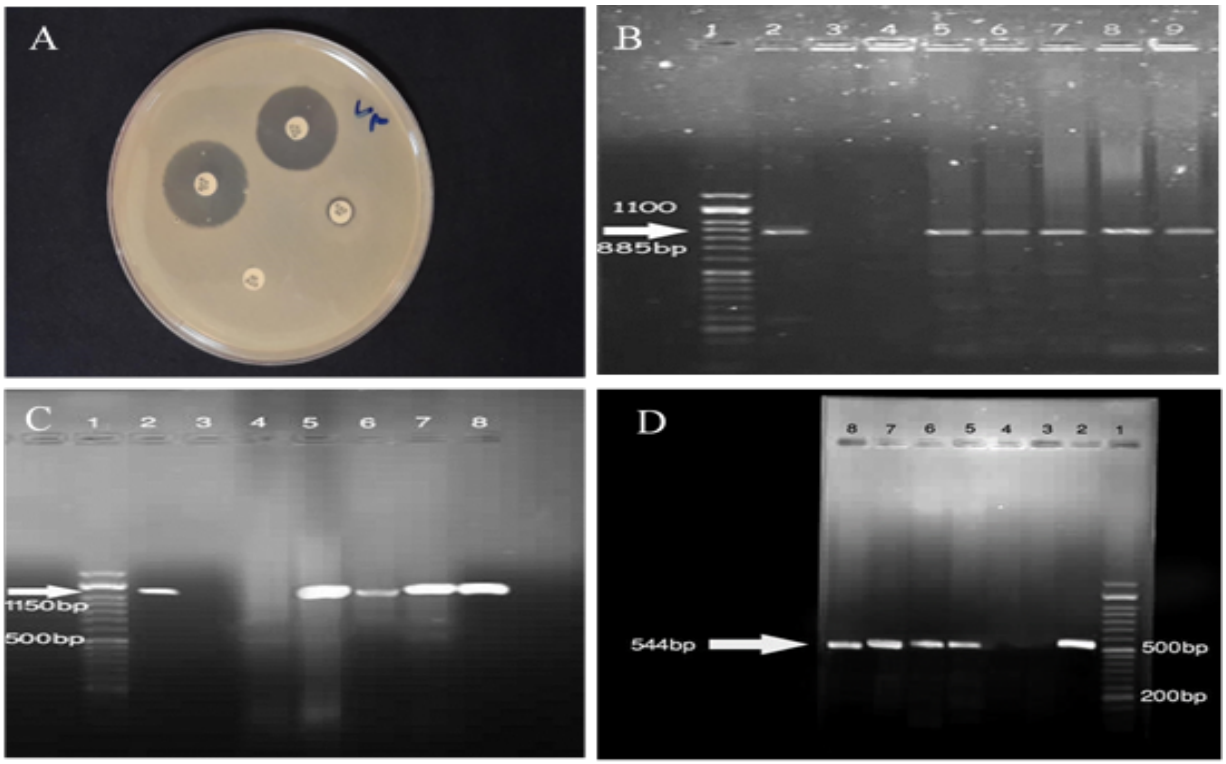

Fig. 1. The Combined Disc Method and ESBL genes amplification product (A) An ESBL Enzyme Producer Isolate; (B) *BlaSHV*- gene; (C) *blaTEM*- gene (D) *blaCTX*- gene.

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DNA extraction and PCR

Bacterial DNA was extracted by boiling method (26), then PCR was performed according to Table 1 with primers designed for target genes (primers for virulence genes and primers for resistance genes).

Table 1. Primers for virulence genes and primers for resistance genes.

Target		Sequences (5′ to 3′)	Annealing temperature (°C)	Product size (bp)	Reference
Resistance genes primers					
SHV	**F**	CACTCAAGGATGTATTGTG	52	885	(27)
	R	TTAGCGTTGCCAGTGCTCG			
TEM	**F**	ATAAAATTCTTGAAGACGAA	50	1150	(27)
	R	GACAGTTACCAATGCTTAAT			
CTX	**F**	TTTGCGATGTGCAGTACCAG	55	544	(28)
	R	CGATATCGTTGGTGGTGCCA			
Primers of virulence factors					
traT	**F**	GGTGTGGTGCGATGAGCACAG	60	290	(27)
	R	CACGGTTCAGCCATCCCTGAG			
Hly	**F**	AACAAGGATAAGCACTGTTCTGGCT	63	1177	(29)
	R	ACCATATAAGCGGTCATTCCCGTCA			
PAP	**F**	GCAACAGCAACGCTGGTTGCATCAT	60	336	(30)
	R	AGAGAGAGCCACTCTTATACGGACA			
fimH	**F**	CATTCGCCTGTAAAACCGCC	60	207	(27)
	R	ATAACACGCCGCCATAAGCC			
aer	**F**	TACCGGATTGTCATATGCAGACCGT	62	602	(29)
	R	AATATCTTCCTCCAGTCCGGAGAAG			

*All PCR reactions done by following cycles: 5 ′ multiplied at 94 ° C in 1 cycle, 35 ″ at 94 °C; denaturation, 35″ at 52 °C; primer binding, 35 ″ at 72 °C, 40 cycles and 5′ at 72 °C for final Extensions, F: Forward, R: Reverse.

Data analysis

The results were analyzed with SPSS™ software version 21.0 (IBM Corp., Armonk, NY, USA). The results are presented as descriptive statistical data relative frequency. Chi-square ($\chi 2$) or Fisher's accurate tests were used in the analysis. One $P<0.05$ was found to be significant.

Results

One hundred strains of *E. coli* were isolated from patients at Allame-Bohlol Gonabadi Hospital in Gonabad, Iran, and analyzed. Urine samples were from both sex (76% female, 24% male). In Gram staining and morphological examination, gram-negative short rod bacteria were seen singly or in pairs. The bacteria had grown in both blood Agar and EMB Agar. Metallic polishing was evident on the EMB medium. In biochemical studies, all isolates included in our study were positive for indole, motility, and methyl red (MR) positive, and for Voges Proskauer (VP) and Simon Citrates were reported negatively. In this study, in isolated strains, the highest resistance was seen to ampicillin 72%, trimethoprim/sulfamethoxazole (56%), and tetracycline (56%). Resistance to other antibiotics is as follows: tobramycin (13%), cefoxitin (11%), cefotaxime (31%), ciprofloxacin (26%), chloramphenicol (18%), cefuroxime (29%), gentamicin (12%), cefazolin (39%), colistin (2%), cefixime (35%), ceftriaxone (31%), nitrofurantoin (5%),

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doripenem, and meropenem (0%) (Table 2). Out of one hundred strains of *E. coli*, 30 samples (30%) were identified as ESBL. By gender, 63% were seen in women and 27% in men. Amongst the isolates tested, the seven strains certainly had all three of the disaster resistance genes. In ESBL-positive *E. coli* isolates, the highest resistance to beta-lactam antibiotics was seen (Table 3). 84% isolates had bla_{CTX}, 52% had $bla_{TEM,}$ and 7% had bla_{SHV}. The lowest beta-lactamase gene detected was $bla_{SHV,}$ and the highest was bla_{CTX}. The frequencies of $bla_{SHV,}$ bla_{TEM} and bla_{ctx}beta-lactamase genes among ESBL positive isolates were 11.1%, 48.1%, and 93.3%, respectively. Finally, the prevalence of four virulence factors in 100 UPEC isolates studied was investigated. Also, PCR products of *pap, traT, aer*, *hly* and f*im* genes and ESBL genes after electrophoresis on agarose gel are shown in Figure 1 and 2. According to the virulence determinants, the *traT* gene (94%) is the most common virulence gene. Afterwards, *fim* gene was found in 90 (90%) cases. Then, respectively, *hly* (21%), *are* (82%), and *pap* (91%) genes were positive, so amongst the studied isolates, *tatT* and *fim* gene was the most common.

Table 2. Response of E. coli-isolates recovered from UT system specimens to tested antibiotics (N=100).

No.	Antibiotics	Concentration (µg)	Resistance (%)	Intermediate (%)	Sensitive (%)
1	Ceftazidime	30	27	5	68
2	Colistin	25	2	1	97
3	Cefixime	5	35	3	62
4	Doripenem	10	0	0	100
5	Ceftriaxone	10	31	2	67
6	Tetracycline	30	56	1	43
7	Cefazolin	30	39	26	35
8	Ofloxacin	5	23	0	77
9	Cefotaxime	30	31	2	67
10	Imipenem	10	0	0	100
11	Ampicillin/sulbactam	10	12	5	83
12	Gentamicin	10	12	1	87
13	Chloramphenicol	30	18	2	80
14	Cefoxitin	30	11	1	88
15	Ertapenem	10	0	3	97
16	Piperacillin/tazobactam	100	5	5	90
17	Ciprofloxacin	5	26	1	73
18	Trimethoprim-sulfamethoxazole	1.25	56	3	41
19	Ampicplin	10	72	2	26
20	Meropenem	10	0	0	100
21	Tobramycin	10	13	8	79
22	Cefuroxime	30	29	5	69
23	Nitrofurantoin	300	5	0	95

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Table 3. ESBL+ E. coli strains response to tested antibiotics (N=30).

No.	Antibiotics	Concentration (µg)	Resistance (%)	Intermediate (%)	Sensitive (%)
1	Nitrofurantoin	300	6.7	3.3	90
2	Tetracycline	30	76.7	3.3	20
3	Ceftazidime	30	73.4	3.3	23.3
4	Gentamicin	10	36.6	0	63.4
5	Ciprofloxacin	5	50.1	6.6	43.3
6	Meropenem	10	0	0	100
7	Chloramphenicol	30	26.6	3.4	70
8	Ceftriaxone	10	96.7	3.3	3.3
9	Ertapenem	10	0	7.4	92.6
10	Colistin	25	0	0	100
11	Ampicillin	10	100	0	0
12	Trimethoprim-sulfamethoxazole	1.25	73.2	3.4	23.3
13	Cefuroxime	30	96.4	3.3	0
14	Tobramycin	10	44	9.4	46.6
15	Cefazolin	30	100	0	0
16	Doripenem	10	0	0	100
17	Piperacillin/tazobactam	100	10.2	13.2	76.6
18	Ampicillin/sulbactam	10	36.6	3.4	60
19	Cefixime	5	96.7	3.3	0
20	Cefotaxime	30	96.7	3.3	0
21	Cefoxitin	30	16.7	10	73.3
22	Imipenem	10	0	0	100
23	Ofloxacin	5	50.1	6.6	43.3

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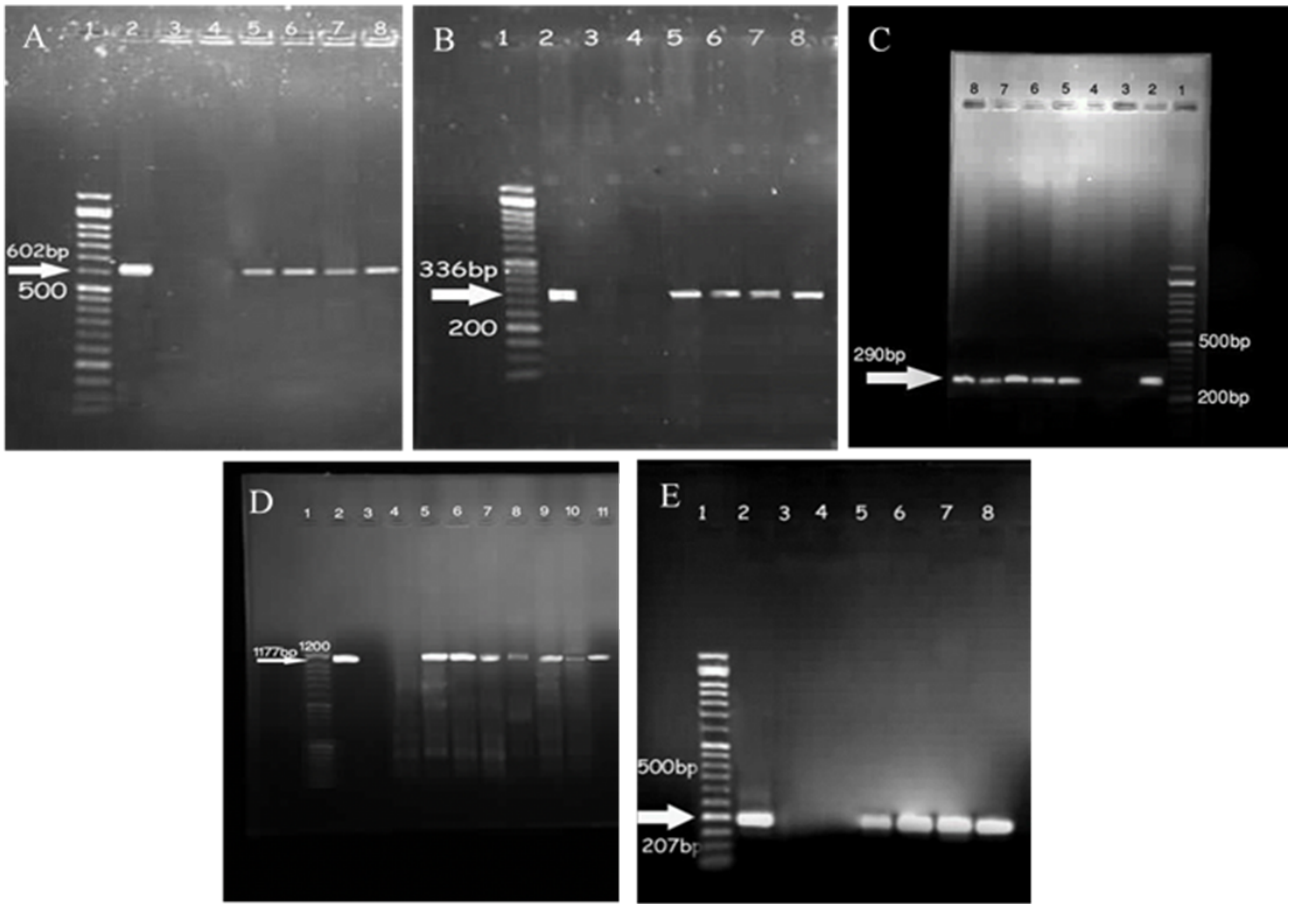

Fig. 2. Pathogenic genes amplification product. (A) aer-gene; (B) pap-gene; (C) traT-gene; (D) hly-gene; (E) fimH-gene.

Discussion

Determining drug resistance allows physicians to use appropriate and effective antibiotics. In this way, the risk of developing resistant strains is reduced, and there will be no unpleasant side effects of using ineffective antibiotics, and treatment will be successful. UTIs are a worldwide health problem, with *E. coli* being the most common etiology. *E. coli* strains of UTI virulence factor express several factors (31, 32). This study examined the drug resistance frequency in *E. coli* causing UT system infections in hospital patients and exploring its virulence factors**.**

In 2017, Keikha et al. reported the highest rates of resistance to ceftazidime (44.8%), nitrofurantoin (26.1%) and imipenem (4.5%) (33). The different results of the study may be as a result of regional distance, level of community health and arbitrary use of antibiotics. As well as, in most urinary tract infections, experimental therapy is began before acquiring results culture of urine specimen in the microbiology laboratory. As a result, antibiotic resistance may increase due to repeated and erroneous consumption of antimicrobial drugs in uropathogenic organisms. It has been shown that there is no significant difference between individuals' sex and age and resistance incidence. bla_{CTX} gene was isolated from 82 cases (92.5%), bla_{TEM} gene from 52 cases (48.1%) and bla_{SHV} gene from 7 cases (11.1%). In a study in 2010 by Masrour and co-workers conducted in Pakistan, the bla_{CTX} gene had the highest prevalence of 57.7% out of 121 *E. coli* samples, followed by *a blaTEM gene* prevalence of 20.3% bla_{SHV} gene prevalence of 15.4% has been reported (34). The selective pressure can be the reason emerging of high ratio of ESBLs producing strains. This particular strain may be due to the wide consumption of antibiotics, genomic diversities in virulent clones, high transmission potential, and extended hospital stays. In the

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current investigation, the production of broad-spectrum beta-lactamases was identified in a high percentage of urinary *E. coli* strains. This indicates that resistance to different antibacterial varies according to treatment regimens in other area. In many regions in worldwide, the emergence of ESBL-producing isolates is spreading (35, 36). The traT protein has importance in the resistance of bacteria to the lethal effect of serum. The outcomes of the current research indicated that 94% of UPEC isolates contain *traT* gene. Oliveira et al. also reported that 76% of urine samples infected with multidrug-resistant bacteria carry the *traT* gene (37). These data are consistent with the results of our study, and suggest *traT* can be considered a target for therapeutic interventions. The attachment of *E. coli* to the urinary tract cells is mediated by bacterial ligands attached to the carbohydrates of the host cell wall making it resistant to urine flow and bladder emptying (38). In our study, the existence of *fimH* gene was defined by PCR in 90% of the isolates. Tarchuna et al. reported that among the virulence genes of the UPEC strain, the *fimH* gene is the most common and is found in 68% of UTI isolates (31). Garofalo et al. and Watts et al. also confirmed this finding in their studies (39, 40). Another common virulence factor in our study was the *aer* gene (82%). This gene was present in more than 50% of *E. coli* isolates in Tunisia, Romania, and Iran (41, 42). Santo nad coworkers et al. also reported a rate of 76% for *aer* among the 100 *E. coli* strains in Brazil (43). In current research, the *fimH* and *aer* genes were present in more than 50% of the isolates, indicating the importance of these two genes in the invasive power of UPEC. Due to the differences in studies on the prevalence of virulence factors and resistance factors, it shows that the prevalence of the genes are different in different regions and there is need for further studies to localize the appropriate treatment for urinary tract infection for each geographical region.

Extensive efforts are also needed to provide appropriate treatment guidelines for each area. Based on the outcomes of current study and literature reviews, it can be concluded that in current decades, over-use of antimicrobial has raised bacterial resistance to them, and resistance genes are transmitted faster between virulence bacteria. It is avoidable by study on the frequency of resistance genes and virulence genes in microorganisms and finding a relationship between them. Pathogenic genes may also rise the survival and proliferation of bacteria in the UT system. Detection these virulence genes as the primary controllers of infection can help better illnesses management.

Due to economic, social, health, geographical and environmental conditions between developing and industries countries, more studies should be conducted in developing countries to identify the pathogens of urinary tract infections to determine the pattern of pathogenesis. Limitations include the small number of samples and geographical boundaries, which can also affect drug resistance patterns.

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Conflict of interest

Authors state no conflict of interest.

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