Resistance Investigation of Uropathogenic Escherichia coli strains isolated from urinary tract infections in the north of Iran

Running: Resistance investigation of UPEC isolated from urine

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Abstract

Background: Urinary tract infection (UTI) caused by Uropathogenic *Escherichia coli* (UPEC) is a worldwide health problem. Virulence factors (VFs) expressed by UPEC strains promote the pathogenicity of bacteria in the urinary tract. Treatment of the infection is often difficult due to the high antimicrobial resistance of *E. coli*. This study aimed to determine VFs and the antibiotic susceptibility pattern of isolated UPEC strains in the north of Iran.

Methods: 105 urine samples were collected from females with UTIs, in north of Iran, Rasht. The samples were cultured on Eosin Methylene Blue (EMB) agar and MacConkey agar. The plates were incubated at 37°C for 24 h and the pure isolates were identified using Gram stains and standard biochemical tests. The presence of six VF genes, including *papC*, *sfa/foc*, *fimH*, *afa*, *ibeA*, and *neuC*, was identified by polymerase chain reaction (PCR) in UPEC strains and verified by direct sequencing. Antibiotic susceptibility test (AST) was performed by the disk diffusion method based on the Clinical and Laboratory Standards Institute (CLSI M02) guidelines.

Results: 65.71% isolates were identified as *E. coli*. The most frequent virulence gene was *fimH* (100%), and the least one was *afa* (1.44%). The highest and the lowest antibiotic resistance rates were observed against Cephazolin (66.66%) and Gentamicin (24.63%), respectively. Indeed, the prevalence of multiple drug resistance (MDR) was determined as 73.91%.

Conclusion: Our study highlighted the importance of local monitoring in UPEC isolates due to the high genetic mutation capacity of the pathogen, environmental, and patient properties to recommend the best strategies against UTIs.

Introduction

Urinary tract infection (UTI) is a common disease that is considered a global health problem because of its high morbidity and mortality rates as well as high medical costs (1). Uropathogenic *E. coli* (UPEC) is the main agent causing UTI. Approximately 75-95 percent of uncomplicated UTIs and 40-50 percent of complicated UTIs are caused by UPECs (2-4).

Pathogenicity of UPEC is due to the wide range of virulence factors (VF), including adhesins, toxins, invasins, surface polysaccharides, flagella, and iron-acquisition factors (1, 4). Fimbriae and afimbrial adhesins such as type 1 fimbriae (*fimH*), P fimbriae (*papC*), S fimbriae, and F1C fimbriae (*sfa/foc*) and afimbrial adhesin (*afa*) are the most common adhesins detected in strains isolated from UTI patients (2,5).

FimH protein (mannose-specific adhesins of type I fimbriae) mediates host-pathogen interaction by attachment to the uroepithelial proteins and promoting biofilm formation (2, 6). P-fimbriae, encoded by pilus associated with pyelonephritis (*pap*) operon play an important role in mediating colonization by specific binding to digalactoside containing receptors on epithelial surfaces of intestine, vagina and urinary tract (6, 7). S fimbriae attach to the epithelial and endothelial cells of the lower human urinary tract and help the pathogen to spread within host tissues (2, 4, 8). F1C fimbriae are associated with S-type fimbriae and bind to the β-GalNac-1, 4b-Gal residues on glycolipids expressed by epithelial cells of distal tubules and cells of the collecting ducts of the kidney, as well as by endothelial cells of the bladder and kidneys (2). Afimbrial adhesins are the superficial virulence factors in UPEC strains and bind to the decay-accelerating factor (DAF) receptor expressed on the epithelial cells of the urinary tract. Strains causing pyelonephritis and recurring cystitis are carrying the operon of *afa* family (2, 5, 9).

There are miscellaneous virulence genes in UPEC as *ibeA* (invasion of brain endothelium) and *neuC* (sialic acid biosynthesis) which are less common in these strains (10). IbeA protein promotes invasion into the cells and tissues as well as neuC which is the K1capsule antigen and protects bacteria against phagocytosis and helps them to spread in host. These two genes are most frequent in strains which are responsible for meningitis (5, 8, 10).

The basis of treatment in urinary infections is to choose a suitable antibiotic with wide efficiency and effectiveness. The β-lactams, quinolones and cephalosporins are some of the common antibiotics which are used in over the world (11). However, the studies show the antibiotic resistance of UPEC strains is growing increasingly (3, 9). The existence of multidrug-resistant (MDR) strains of *Escherichia coli* (*E. coli*) makes it difficult to treat the infection. Mutation and acquisition of mobile genetic elements are mechanisms lead to protection of strains against the antimicrobial activity of drugs (3, 12).

Here, the lack of strain characterizations as VFs in Iran, led us to investigate the prevalence of different aforementioned VFs in UPEC strains isolated from patients with UTI in Rasht, a city in north of Iran. Indeed, antimicrobial susceptibility testing (AST) was performed to illustrate the susceptibility/ resistance pattern of the strains to current antibiotics used by clinicians to find the best cure.

Methods

A total of 105 urine samples were collected from females with HA- UTIs and CA- UTIs admitted to the Razi Hospital and Social Security Polyclinic in Rasht, from August, 2017 to July, 2018.

The samples were cultured on *Eosin Methylene Blue agar* (EMB agar) and MacConkey agar. The plates were incubated at 37°C for 24 h and the pure isolates were characterized and identified using Gram-stains and biochemical tests such as triple iron sugar utilization, citrate

utilization, indole production, and methyl red-Voges Proskauer based on the instructions provided by CLSI (13).

The confirmed *E. coli* isolates were cultured in Tryptic Soy Broth (TSB) containing 10-15 percent glycerol and stored at -20°C for further investigations. DNA was extracted using a DNA extraction kit (Tiangen- China) according to the manufacturer's instructions. The quality of extracted DNA was examined by 1% agarose gel electrophoresis.

All media were used as agar and broths were Merck, Germany.

2.2. Detection of virulence genes

Six virulence genes, including *papC*, *sfa/foc*, *fimH*, *afa*, *ibeA* and *neuC* were identified by PCR (8). The specific primers (Takapouzist, Iran) are listed in Table 1. The amplification was done in a total volume of 25 µl containing 20 µl Master Mix (Golden double helix), 20 pmol/l of each primer, and 3 µl (25 ng) of extracted DNA. The reaction conditions performed in a thermal cycler (Bio-Rad, Germany) were as follows: initial denaturation at 94°C for 3 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing (temperature and time for each paired primers are mentioned in Table 1), and extension at 72°C for 1 min, followed by a final 10 min extension period at 72°C. The same volume of each amplified product and the Safe Super Stain (Golden double helix) were mixed and electrophoresed in a 2% agarose gel (Merck, Germany) in 1X Tris-Borate-EDTA (TBE) buffer (Cinnagen), and photographed using a UV transillumination imaging system (Labnet, USA). A 100-bp DNA ladder (Sinaclon) was used to determine the molecular size of the PCR products.

Table 1. Characteristics of virulence gene amplification by PCR

Category	Virulence gene	Primer sequence* (5´-3´)	Amplicon size (bp)	Annealing temperature	Time (seconds)
Fimbrial adhesins	*fimH*	TGCAGAACGGATAAGCCGTGG GTCACCTGCCCTCCGGTA	508	55	60
	papC	GACGGCTGTACTGCAGGGTGTGGCG ATATCCTTTCTGCAGGGATGCAATA	328	65	60
	sfa/foc	CTCCGGAGAACTGGGTGCATCTTAC CGGAGGAGTAATTACAAACCTGGCA	410	65	60
Afimbrial adhesins	*afa*	GCTGGGCAGCAAACTGATAACTCTC CATCAAGCTGTTTGTTCGTCCGCCG	750	65	45
Miscellaneous	*ibeA*	TTACCGCCGTTGATGTTATCA CATTAGCTCTCGGTTCACGCT	171	60	45
	neuC	AGGTGAAAAGCCTGGTAGTGTG GGTGGTACATTCCGGGATGTC	675	61	45

*- Reference: (5)

Sequencing

Two amplified fragments for each gene were confirmed by direct sequencing using the 3730xl DNA analyzer (Macrogen, Korea). To verify virulence genes, BLAST searches for nucleotide and protein sequence alignments were done using the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and BioEdit software version 7.2.5. In the end, the phylogenetic tree was analyzed using the maximum likelihood (RAxML) model (http://www.trex.uqam.ca) (14).

Antibiotic Susceptibility Test (AST)

Bacterial susceptibility to antibiotics was determined using the disk diffusion method on Mueller-Hilton agar plates (Merck, Germany) based on the Clinical and Laboratory Standards Institute (CLSI) guidelines (13, 15). A few colonies were dissolved in normal saline serum to

reach a 0.5 McFarland standard concentration. Then it was cultured on a Mueller-Hinton agar plate, and antibiotic disks were placed at the standard distance. Plates were incubated at 37°C for 24 h. Finally, the diameter of the inhibition zone developed around each disk was measured. Ten antimicrobial agents (Padtan teb, Iran) from different classes of antibiotics were used including Ceftriaxone (CRO) (30µg), Ceftazidime (CAZ) (30µg), Cefotaxime (CT) (30µg) and Cephazolin (CZ) (30µg) of Cephalosporins, Kanamycin (K) (30µg) and Gentamicin (GM) (10µg) of Aminoglycosides, Ciprofloxacin (CP) (5µg)) of Fluoroquinolones, Chloramphenicol (C) (30µg), Imipenem (IMP) (10µg) of Carbapenems, Tetracycline (TE) (30µg) of Tetracyclines and Chloramphenicol (C) (30µg). An isolate was considered MDR if it was resistant to at least three antibiotics.

Results

From 105 urine samples, 69 (65.71%) isolates were identified as *E. coli*. The frequency of VFs in UPEC isolated strains showed that all strains (100%) had the gene encoding *fim*H. The prevalence of other common fimbrial adhesins followed by 62 (89.85%) for *papC* and 19 (27.53%) for *sfa/fos*, whereas *afa* had the lowest frequency with 1 (1.44%). The frequency of *neuC* and *ibeA* was 17 (24.63%) and 7 (10.14%), respectively. Gel electrophoresis of virulence genes is shown in Figure 1.

The result of alignments shows a high degree of similarity (98-100%) of detected VFs to the submitted nucleotide and protein sequences in GenBank (Table 2).

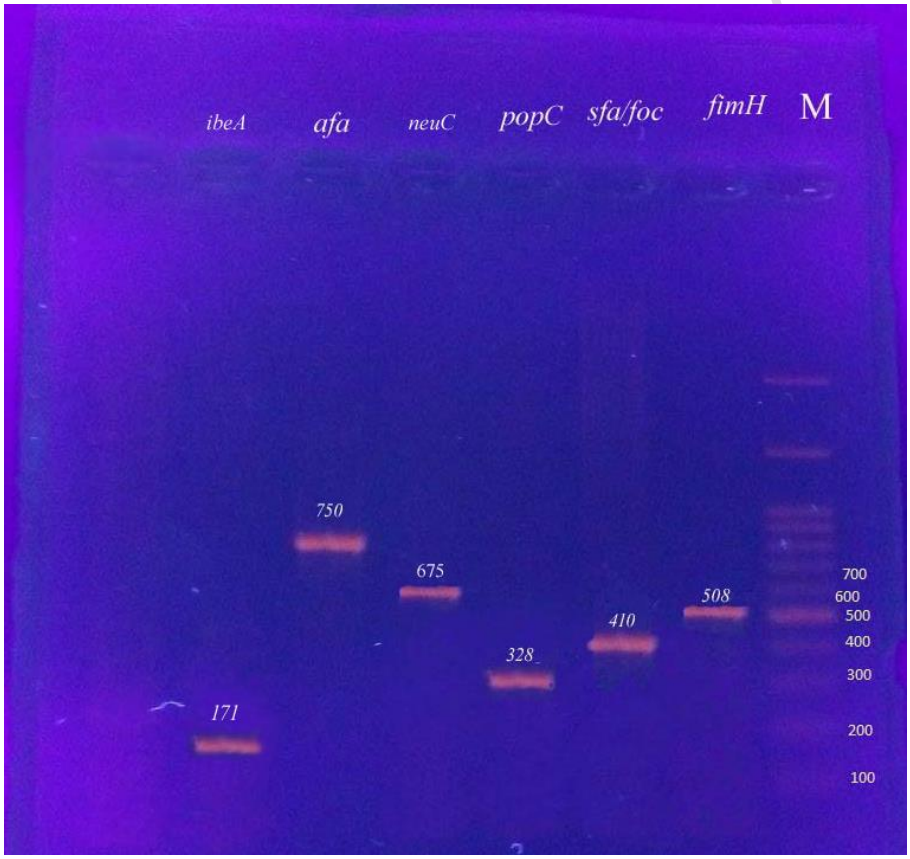

Figure 1. Gel electrophoresis of PCR products of virulence genes. Lane M: ladder (100bp), lane *fim*H (508 bp), lane *sfa*/*foc* (410 bp), lane *pap*C (328 bp), lane *neu*C (675 bp), lane *afa* (750 bp) and lane *ibe*A (171 bp).

The result of alignments shows a high degree of similarity (98-100%) of detected virulence factors to the submitted nucleotide and protein sequences in GenBank (Table 2).

Table 2. The result of 10 Sequences producing significant alignments

Gene	Coverage range (%)		Identity range (%)		Accession number [c]	
	BLASTN[a]	BLASTX[b]	BLASTN	BLASTX	BLASTN	BLASTX
fimH	100	99	100	100	CP046006.1	WP_000326171.1
papC	96-100	96-99	100	99.06-100	X61239.1	QFQ50497.1
sfa/fos	100	57	100	100	CP019243.1	ADX21060.1
afa	100	58	99.86	98.53-100	CP032145.1	AEF32261.1
ibeA	100	89-91	98.84	98.08-100	CP043181.1	QDO72751.1
neuC-1	100	99	99.56-100	99.56-100	CP022730.1	GCS57713.1
neuC-2	100	99	100	99.54-100	CP043950.1	WP_087904218.1

[a] - Search nucleotide databases using a nucleotide query
[b]- Search protein databases using a translated nucleotide query
[c]- Accession number of the first significant alignment

The most homogenous gene was *fimH* with 100% coverage and similarity. Furthermore, based on the sequencing results, two genes of *neuC* were found in isolated UTEC strains labeled with *neuC*-1 and *neuC*-2. The similarity of the two *neuC* genes was 99.54% and both of them were completely similar to the sequences in the GenBank blast search, although the sequences similar to *neuC*-2 were more frequent. The dendrogram of VFs shows three different clusters for adhesins, *neuC* and *ibeA* (Figure 2).

From AST data (Table 3), the highest antibiotic resistance was observed towards Cephazolin, followed by Ceftazidime, Tetracycline, Cefotaxime, Ciprofloxacin, Ceftriaxone, Imipenem, Kanamycin, Chloramphenicol, and Gentamicin (the most susceptible one). Only 2 (2.89%) isolates were susceptible to all antibiotics, whereas 62 (89.85%) isolates were resistant to at least one antibiotic. The rate of MDR was 51 (73.91%) (Table 4). The amount of MDR indicates that the majority of UPEC isolates are resistant to three or more antibiotics.

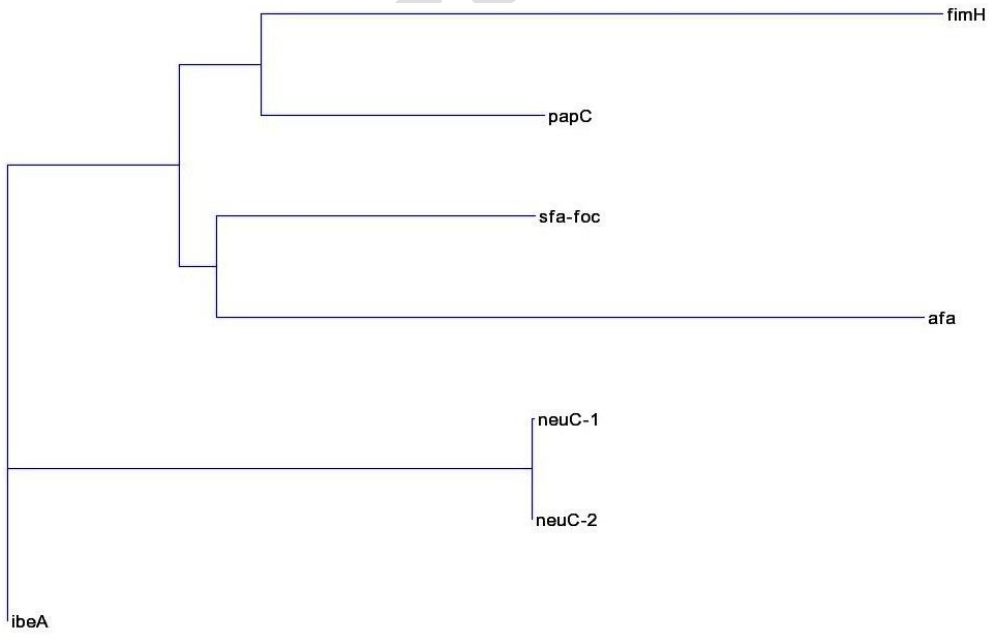

Figure 2. The dendrogram of virulence genes, using the Randomized Axelerated Maximum Likelihood (**RAxML**) method.

Table 3. Antibiotic susceptibility pattern of 69 UPEC isolates

Antibiotic	Resistance no. (%)	Intermediate no. (%)	Susceptible no. (%)
Gentamicin	17 (24.63)	0 (0)	52 (75.36)
Kanamycin	22 (31.88)	13 (18.84)	34 (49.27)
Chloramphenicol	22 (31.88)	4 (5.79)	43 (62.31)
Imipenem	29 (42.02)	8 (11.59)	32 (46.37)
Ciprofloxacin	39 (56.52)	6 (8.69)	24 (34.78)
Tetracycline	41 (59.42)	1 (1.44)	27 (39.13)
Ceftriaxone	30 (43.47)	3 (4.34)	33(47.82)
Cefotaxime	40 (57.97)	3 (4.34)	26 (37.68)
Ceftazidime	44 (63.76)	8 (11.59)	17 (24.63)
Cephazolin	46 (66.66)	12 (17.39)	11 (15.94)

Table 4. Pattern of Multi-Drug Resistance in UPEC isolates

Number of Multi-Drug-Resistant Antibiotics	Incidence rate (%)
three	5 (7.24)
Four	12 (17.39)
five	7 (10.14)
six	4 (5.79)
seven	3 (4.34)
eight	10 (14.49)
nine	8 (11.59)
ten	2 (2.89)
Total of MDR isolates	51 (73.91)

Discussion

Genetic investigations indicate pathogenic procedures and provide reasonable strategies against the infection. VFs encoded on plasmids or on "pathogenicity-associated islands" (PAIs) mediate colonization and persistence of bacteria in the urinary tract. They are suggested to be primarily inherited vertically or to be transferred horizontally between lineages (16).

The prevalence of VFs and the antibiotic resistance rate in UPEC strains isolated in this study were compared to similar studies carried out in different cities of Iran in the last decade (Table 5).

Here, the details of the UPEC population isolated from urine samples of women suffering from UTI in the north of Iran (Rasht, Guilan Province) were evaluated. Based on our findings, the most predominant virulence gene was *fimH* (100%), followed by *papC* (89.85%), *sfa/foc* (27.53%), *neuC* (24.63%), *ibeA* (10.14%), and *afa* (1.44%).

Pointing to table 5, the high frequency of *fimH* is in agreement with most previously published data performed in different cities of Iran and other countries (1, 17, 18). *PapC*, *sfa/foc*, and *afa* are other verified common VFs that exist in UTEC strains; however, their prevalence varied due to the differences related to various populations and different geographical regions where UTEC isolates were identified. A few studies looked for *neu*C and *ibe*A in UPEC strains and confirmed their low prevalence in UPEC strains.

Whereas some VFs are common in several pathotypes of extraintestinal pathogenic *E. coli* (ExPEC) like *fimH* and *papC*, some are specialized for UPECs, such as *afa* and *foc* as well as some others which are not present in UPEC strains, including K1 capsular antigens and the *neuC* gene and or the *ibeA* invasion gene found in neonatal meningitis *E. coli* (NMEC) and

sepsis-associated *E. coli* (SEPEC) (2, 5, 8). So, the presence of these genes in UPECs is a question considering their functions in promoting biofilm formation and invasion. According to a study carried out by Najafi et al., there are eight phylogenetic groups (B1, B2, F, D, E, Clade I, C, and A) of UPEC strains, while a group of unclassified strains is still remains. *FimH* is found in all phylogroups except A. *PapC* was absent in B1 and A phylogroups. *Afa* was present in B2, E, D, and unclassified phylogroups. *Sfa* was just in the unclassified group. *NeuC* was present in three phylogroups (B2, C and unclassified group), and *ibeA* was detected in B2 and unclassified phylogroups. Therefore, the findings support the genetic recombination and geographic distribution of new phylogenetic groups of UPEC (10).

Based on Table 5, the extremely high percentage of UPEC strains isolated in different cities of Iran, as observed in Rasht, showed an MDR phenotype. It is obvious for researchers all over the world that a high rate of antibiotic resistance occurs in UPEC isolates (11). According to the most recent antibiotic resistance surveillance system (GLASS) report published by the WHO in 2016-2017, Iran is one of the ten countries enrolled in GLASS in the eastern Mediterranean region. Resistance in *E. coli* develops either through mutation or by acquisition of mobile genetic elements, as in fluoroquinolone, penicillin, and third-generation cephalosporin resistance. Resistance to third-generation cephalosporins generally confers resistance to several other antibacterial drug classes by producing enzymes known as extended-spectrum β-lactamases (ESBLs) in *E. coli* strains (12). Here we analyzed the antimicrobial resistance of isolated UPEC strains to six classes of antibiotics, including Aminoglycosides, Cephalosporins (first and third-generation), Chloramphenicol, Carbapenems, Fluoroquinolones and Tetracyclines. In accordance to results, if based on guidelines for UTIs, resistance level >20% was used as a cut-off (19), Kanamycin, Chloramphenicol, Imipenem, Ceftriaxone, Ciprofloxacin, Cefotaxime, Tetracycline, Ceftazidime and Cephazolin with the resistant rate of 22 (31.88%), 22 (31.88%), 29 (42.02%), 30 (43.47%), 39 (56.52%), 40 (57.97%), 41 (59.42%), 44 (63.76%) and 46 (66.66%) should not be recommended against UTIs and Gentamicin with the least resistance rate, 17 (24.63%) and the most susceptibility rate, 52 (75.36%) was the only antibiotic suggested for treatment. The results are approximately similar to other studies in Iran and even in all countries mentioned in GLASS, with a significant exception, Imipenem. Whereas the resistance rate to the third-generation of Cephalosporins and Ciprofloxacin is at a high level, and Imipenem is introduced as one of the most susceptible antibiotics against UTEC strains, in Rasht and Abadan (north and south of Iran) the resistant rate to Imipenem was alarming, and clinicians in these cities should be aware. Taken together, since the antibiotic susceptibility pattern varies in different populations and geographical regions, it is crucial to obtain the potency of isolated pathogens against current antibiotics to ensure the treatment.

Table 5. An epidemiological study (comparing the prevalence of virulence factors and antibiotic resistance rate in UPEC strains isolated in this study to similar studies carried out in different cities of Iran in the last decade)

City	Time	No.	MDR (%)	Antibiotic resistance (%)									Prevalence of VFs (%)						Ref
				CZ	CAZ	CT	CRO	TE	CP	IMP	C	GM	*ibeA*	*neuC*	*afa*	*sfa/ foc*	*papC*or *pap*	*fimH*	
Rasht (north)	2012	33	100	-	51.51	60.6	57.57	81.81	33.33	33.33	45.45	-	-	-	-	-	-	-	(20)
	2013	110	-	-	41.8	-	-	60	43.6	-	-	50.9	-	-	-	-	-	-	(21)
	2018	69	73.91	66.66	63.76	57.97	43.47	59.42	56.52	42.02	31.88	24.63	10.14	24.63	1.440	27.53	89.85	100	*
Tehran (capital)	2012	121	100	-	22.7	13.6	-	69.6	15.1	1.5	-	16.6	-	-	-	-	-	71.2	(22)
	2012	60	-	-	-	-	-	-	-	-	-	-	-	-	26.66	30	70	-	(23)
	2012	105	-	-	-	-	-	-	-	7.61	-	-	-	-	38.09	50.47	40	39.04	(24)
	2014	156	-	-	35.9	9.6	41	60.3	32.7	0	-	17.3	-	-	-	-	-	-	(25)
	2015	100	-	-	-	-	-	-	-	-	12	21	-	-	-	-	-	-	(26)
	2015	147	-	-	31.97	-	20.4	-	87.75	4.08	-	95.91	-	-	83.67	89.79	-	95.23	(27)
	2016	60	100	50	-	-	-	50	34	0	-	19	-	-	-	-	-	-	(28)
Shiraz (south)	2012	85	82.35	-	-	68.2	-	-	-	-	-	-	9.4	-	-	-	-	34.1	(29, 30)
	2016	126	77.8	-	65.1	-	-	-	55.6	0.8	-	19.8	-	-	46	79.4	-	99.2	(31)
	2017	121	-	-	66.9	-	-	67.8	58.7	22.3	-	35.5	-	-	-	-	-	98.3	(32)
Kerman (south east)	2009	137	-	71	-	-	-	-	29.18	0	-	36.45	-	-	-	35.76	18.97	-	(33)
Urmia (north west)	2012	25	96	-	-	-	-	65	52	-	-	65	-	-	-	-	-	-	(34)
Kashan (central)	2013	150	74	-	49.3	-	56.7	-	61.3	0.7	-	40	-	-	0	0	16.66	-	(35, 36)
Isfahan (central)	2013	135	63	-	55	47	-	-	45	-	-	16	-	-	-	-	-	-	(19)
Kermanshah (west)	2013	200	-	-	-	-	73.5	-	42.9	0	-	30.6	-	-	18.36	18.36	18.36	-	(37)
Bushehr (south)	2013	140	-	-	-	-	-	-	-	-	-	-	3.6	9.3	10.7	0.7	38.6	85	(10)
Zabol (south east)	2013	100	-	74	55	65	-	-	43	-	-	19	67	-	12	81/16	57	95	(38-40)
Ahvaz (south west)	2014	232	-	-	21.98	-	11.63	6.46	23.27	6.46	-	6.03	-	-	-	6.4	44.8	95.7	(41)
Sanandaj (west)	2015	32	-	-	40.6	65.6	-	62.5	43.7	6.2	-	37.5	-	-	15.6	-	25	-	(42)
Yasouj (south west)	2017	130	-	-	-	46.9	46.9	60.2	38.8	1	-	9.2	-	-	-	29	50	-	(43)
Abadan (south)	2018	100	-	-	-	70	50	91	78	59	-	-	-	-	-	-	-	-	(44)

* This study
- Was not detected

Conclusion

Based on our findings, Resistant to first and third generation of Cephalosporines (Cephazolin, Ceftazidime, Cefotaxime and Ceftriaxone) and Tetracyline, is in a high level through the years. Whereas Imipenem is the most susceptible antibiotic, however it approaches to resistance in recent years. The rate of resistance for Ciprofloxacin fluctuates through different years and cities, but is tended to be resistant mostly. Contrariwise Gentamicin is mostly tended to be susceptible. The distribution of VFs reveals the frequency of *fimH* is usually dominant in UTEC population, independent of time and place whereas frequency of other adhesins (*papC*, *sfa/fos* and *afa*) varies between low and high. Moreover, for *neuC* and *ibeA*, studies show low percentage of UPEC isolates carrying these miscellaneous virulence genes.

Overall, VF profile and antimicrobial susceptibility pattern of UPEC strains can vary according to the epidemiological status. So, considering the high genetic mutation rate in *E. coli*, knowledge about the local pathogens helps to select the most effective medical strategies against the infections.

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Conflict of interest

The authors declare no conflicts of interest.

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Ethical approval

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Author contributions

T.Shafighi: Conceptualization. T.Shafighi, Z.Askari and Z. Mirzapour: Methodology. T.Shafighi, Z. Askari, Z. Mirzapour and R.Ghorbanpour: Formal analysis and investigation. T.Shafighi and R. Ghorbanpour: Editing.and Writing Original Draft Preparation. T.Shafighi: Supervision.

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