JOURNAL OF PURE AND APPLIED MICROBIOLOGY, March 2015. Vol. **9**(1), p. 565-572

Uropathogenic *Escherichia coli* in Diabetic Patients of Iran; Virulence Factors and Antibiotic Resistance Properties

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(Received: 18 October 2014; accepted: 06 December 2014)

From a clinical prospective, it is important to know the distribution of virulence factors and antimicrobial resistance properties of Uropathogenic *Escherichia coli* isolated from diabetic patients with severe urinary tract infections. Therefore, the present study was carried out in order to reach above goals. Totally, 110 urine samples were collected from diabetic patients hospitalized due to UTIs. Samples were cultured and those that were *E. coli* positive were subjected to PCR assays to study the distribution of virulence factors. Simple disk diffusion was carried out to study the antibiotic resistance pattern of *E. coli* isolates. We found that 33 out of 110 diabetic urine samples (30%) were positive for *E. coli*. *E. coli* strains of diabetic patients had the highest levels of resistance to tetracycline (87.8%), ampicillin (84.8%), gentamycin (84.8%), ciprofloxacin (75.7%) and cephalothin (54.5%). In the other hand, imipenem and mezlocillin were the best choices for treatment of the cases of UTIs in diabetic patients. *Sfa* (45.4%), *fim* (45.4%) and *afa* (36.3%) had the highest incidence among virulence factors while, *usp* (3.0%), *sigA* (6.0%) and *iha* (6.0%) had the lowest incidence. Conclusions: **Virulent strains of uropathogenic *E. coli* were predominant cause of UTIs in diabetic patients of Iran. High hygienic care, treatment of diabetes and using from disk diffusion method can reduce the dangerous risk of uropathogenic *E. coli* in diabetic patients.**

Key words: Diabetic patients, Urinary tract infections, Uropathogenic *Escherichia coli*, Virulence factors, Antimicrobial susceptibility

Urinary Tract Infections (UTIs) are frequent bacterial infections worldwide. It is estimated that ~ 150 million cases of UTIs occur worldwide[1].The Uropathogenic *Escherichia coli* (*E. coli* (UPEC)) strains are the most common cause of the UTIs (1). UPEC strains show certain virulent properties, including adhesins, iron uptake systems and synthesis of cytotoxins. All of these properties contribute to colonization and invasion of the bacterium to host cells[1, 2].

Aerobactin (*aer*), type 1 fimbriae, P fimbriae (*pap*), hemolysin (*hly*), S fimbriae (*sfa*), afimbrial adhesin I (*afa*I), adhesins, fimbriae, *astA*, *usp*, *set-1*, *iha*, group II capsule synthesis; *sfa*/*foc*, S and *F1C* fimbriae; *traT*, *iutA*, serum resistance; and *fimH*, are known to be involved in pathogenisity of this UPEC strains[1, 3].

A number of investigators have observed that both common and rare infections are reported more frequently among patients with diabetes mellitus (DM)[4, 5]. Studies showed that DM is a risk factor for occurrence of UTIs caused by UPEC strains[4, 5].

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There is global concern due to the high rates of resistance to antimicrobial agents used in the treatment of infections caused by UPEC strains. Several studies have shown that antibiotic resistance in UPEC strains has increased over time[1, 6]. In keeping with this, an epidemiological investigations represented that there was a high incidence of resistance in the UPEC strains of Iranian hospitals (20–100%) to commonly used antibiotics[1,6].

In addition to the lack of adequate surveillance information in patients, there has been a lack of isolate characterization (virulence factors and antimicrobial resistance properties) of UPEC strains among the Iranian patients with DM. Therefore, the present investigation was carried out to study the distribution of virulence factors and antibiotic resistance properties of UPEC strains isolated from the urine samples of diabetic patients of Iran.

MATERIALS AND METHODS

Samples and bacterial isolation

From March to august 2014, a total of 110 urine samples were collected hospitalized diabetic patients suffered from UTIs. Presence of UTIs in pediatrics was confirmed using the ultrasound technique based on the method described previously by MacKenzie *et al*. (1994) (7). All of these urine samples were collected from midstream using the Suprapubic Aspiration (SPA) method based on the standard technique of NICE (2007) (8).

Totally, 3 mL of each sample was blended with 225 mL of nutrient broth (Merck, Germany) for 2 min at normal speed, using a Stomacher lab blender and incubated at 37 °C for 24 h. A 1 mL sample of the nutrient broth culture was mixed with 9 mL of MacConkey broth (Merck, Germany) and further incubated at 37 °C for 24 h. One loop of each tube was streaked on MacConkey agar (Merck, Germany). A typical purple colony of *E. coli* was streaked on *Eosin Methylene Blue agar* (EMB agar; Merck, Germany) plate and incubated at 37 °C for 24h. Green colonies with a metallic luster were considered as typical *E. coli* colonies. Such colonies were confirmed as *E. coli* using standard biochemical tests (e.g., Methyl red, Voges-Proskauer, Indole, and Citrate utilization tests). *E. coli* isolates were stored in Tryptic Soy Broth (TSB, Merck, Germany) containing 20% glycerol at "70°C for further characterization.

Antimicrobial susceptibility testing

Pattern of antimicrobial resistance was examined using the simple disk diffusion technique. The Mueller–Hinton agar (HiMedia Laboratories, Mumbai, India, MV1084) medium was used for this purpose. Antibiotic resistance of UPEC strains against 15 commonly used antibiotics was determined using the instruction of Clinical and Laboratory Standards Institute guidelines (9). Susceptibility of *E. coli* isolates were tested against Ampicillin (10 u/disk), tetracycline (30 µg/disk), gentamycin (10 µg/disk), amikacin (30 u/disk), imipenem (30 u/disk), mezlocillin (30 u/disk), piperacillin (30 µg/disk), cefotaxime (30 µg/disk), ciprofloxacin (5 µg/disk), norfloxacin (30 µg/disk), cotrimoxazole (30 µg/disk), ceftazidime (30 µg/disk) and ofloxacin (5 µg/disk) antibiotic agents (Oxoid). All of the inoculated plates were aerobically incubated at 37 °C for 18-24 h in an aerobic atmosphere. Results were interpreted based on the instruction provided by CLSI (2012) (9). In all reactions, the *E. coli* ATCC 25922 was used as quality control organisms.

DNA extraction and bacterial confirmation using Polymerase Chain Reaction (PCR)

Bacteria were cultured overnight on Nnutrient broth (Merck, Germany) and genomic DNA was extracted from typical colonies using the DNA extraction kit (Fermentase, Germany) according to manufacturer's instruction. All *E. coli* colonies were also confirmed using the PCR technique based on the method described by Li *et al*. (2010) (10). Briefly, a PCR method was done with a total volume of 50 µL including 2 mM $Mgcl_2$, 1 µM of forward primer, 1 µM of reverse primer (specified for the 16S rRNA gene of the *E. coli*) (Table 1), 5 µL PCR buffer 10X, 200 µM dNTP (Fermentas), 1 U Taq DNA polymerase (Fermentas) and 2.5 µL DNA template. The DNA was then amplified by 31 successive cycles of denaturation at 95°C for 45 s, primer annealing at 59°C for 60 s, and DNA chain extension at 72°C for 60 s.

Detection of UPEC virulence factors

Several PCR reactions were used for detection of virulence factors and antimicrobial resistance genes in UPEC isolates. List of primers used for detection of virulence genes and antibiotic

resistance genes is shown in Table 1 (1). PCR conditions are shown in Table 2.

Gel electrophoresis

The PCR products were analyzed by electrophoresis (120 V/208 mA) in 1.5% agarose gel and stained by ethidium bromide. A molecular weight marker with 100 bp increments (100bp ladder, Fermentas, Germany) and 1 kbp increments (1000bp ladder, Fermentas, Germany) was used as size standard. All runs included a negative DNA control consisting of PCR grade water and positive strains of *E. coli* were used as positive controls.

Statistical Analysis

The data were analyzed using SPSS (Statistical Package for the Social Sciences) software and *P* values were calculated using Chi-square and Fisher's exact tests to identify statistically significant relationships between the distribution of virulence genes and antibiotic resistance properties of the UPEC strains isolated from diabetic patients. A P value < 0.05 was considered statistically significant.

Ethical issues

The present study was authorized by the ethical committee of educational hospitals of Tehran, Iran. All patients or their parents signed the written informed consent.

RESULTS

Our work has identified a large numbers of UPEC strains in the urine samples of diabetic patients. Of 110 urine samples studied, 33 samples (30%) were positive for UPEC strains. Table 3 represents the incidence of antibiotic resistance in the UPEC strains of diabetic patients. UPEC strains of our study harbored the highest levels of resistance against tetracycline (87.8%), ampicillin (84.8%), gentamycin (84.8%), ciprofloxacin (75.7%) and cephalothin (54.5%). In the other hand, UPEC strains had the lowest incidence of resistance against imipenem (3%) followed by mezlocillin (24.2%). Statistically significant differences were seen amongst the incidence of bacterial resistance

Table 1. Oligonucleotide primers for detection of virulence genes and *16S rRNA* gene of Uropathogenic *Escherichia coli* isolated from diabetic patients of Iran.

Target genes	Sequence	Size (bp)
set-1	GTGAACCTGCTGCCGATATC ATTTGTGGATAAAAATGACG	147
astA	ATGCCATCAACACAGTATAT GCGAGTGACGGCTTTGTAGT	110
sigA	TCCTCGGTATTATTTTATCC CGTAACCCCTGTTGTTTCCAC	408
papGI	TCGTGCTGAGGTCCGGAATTT TGGCATCCCCCAACATTATCG	461
papGII	GGGATGAGCGGGCCTTTGAT CGGGCCCCCAAGTAACTCG	190
papGIII	GGCCTGCAATGGATTTACCTGG CCACCAAATGACCATGCCAGAC	258
fim	GAGAAGAGGTTTGATTTAACTTATTG AGAGCCGCTGTAGAACTGAGG	559
iha	CTGGCGGAGGCTCTGAGATCA TCCTTAAGCTCCCGCGGCTGA	827
usp	ACATTCACGGCAAGCCTCAG AGCGAGTTCCTGGTGAAAGC	440
sfa	CTCCGGAGAACTGGGTGCATCTTAC CGGAGGAGTAATTACAAACCTGGCA	410
afa	GCTGGGCAGCAAACTGATAACTCTC CATCAAGCTGTTTGTTCGTCCGCCG	750
E. coli 16S rRNA	AGAGTTTGATCMTGGCTCAG CCGTCAATTCATTTGAGTTT	919

to tetracycline and imipenem (P =0.017), ampicillin and cephalothin (P =0.029) and gentamicin and ceftazidime (P =0.029).

Gel electrophoresis of PCR products for virulence genes are shown in figure 1-3.Table 4 represents the distribution of 11 important uropathogenic virulence genes in the UPEC strains of diabetic patients. We found that the most commonly detected virulence factors in the UPEC strains of diabetic patients were *sfa* (45.4%), *fim* (45.4%) and *afa* (36.3%). The incidence of *papGI*, *papGII* and *papGIII* virulence genes were 30.3%, 30.3% and 27.3%, respectively. Lowest incidence of virulence factors were determined for *usp* (3.0%), *sigA* (6.0%) and *iha* (6.0%). We found statistically significant association between the incidence of

Table 2. PCR conditions for detection of virulence genes in Uropathogenic *Escherichia coli* isolated from diabetic patients of Iran

Gene	PCR program	PCR volume (50 µL)
usp	1 cycle:	5 µL PCR buffer 10X
	94 °C - 2 min.	2 mM Mgcl2
	30 cycle:	200 µM dNTP (Fermentas)
	94 °C - 30 s	0.4 µM of each primers F & R
	58 °C - 30 s	1 U Taq DNA polymerase (Fermentas)
	73 °C - 30 s	3 µL DNA template
	1 cycle:	
	72 °C - 10 min	
iha	1 cycle:	5 µL PCR buffer 10X
	94 °C -6 min.	1.25 mM Mgcl2
	30 cycle:	150 µM dNTP (Fermentas)
	94 °C - 45 s	1 µM of each primers F & R
	58 °C - 60 s	1.2 U Taq DNA polymerase (Fermentas)
	72 °C - 75 s	3 µL DNA template
	1 cycle:	
	72 °C - 8 min	
papGI, papGII, papGIII	1 cycle:	5 µL PCR buffer 10X
	95 °C - 2 min.	1.25 mM Mgcl2
	30 cycle:	100 µM dNTP (Fermentas)
	94 °C - 60 s	1 µM of each primers F & R
	69 °C - 30 s	1.5 U Taq DNA polymerase (Fermentas)
	72 °C - 2 min	3.5 µL DNA template
	1 cycle:	
	72 °C - 10 min	
fim	1 cycle:	5 µL PCR buffer 10X
	94 °C - 3 min.	1.25 mM Mgcl2
	40 cycle:	125 µM dNTP (Fermentas)
	94 °C - 60 s	0.5 µM of each primers F & R
	58 °C - 70 s	1.2 U Taq DNA polymerase (Fermentas)
	72 °C - 70 s	3 µL DNA template
	1 cycle:	
	72 °C - 6 min	
set1, astA, sigA	1 cycle:	5 µL PCR buffer 10X
	94 °C - 3 min.	2 mM Mgcl2
	30 cycle:	150 µM dNTP (Fermentas)
	94 °C - 30 s	0.5 µM of each primers F & R
	55 °C - 60 s	1 U Taq DNA polymerase (Fermentas)
	72 °C - 60 s	3 µL DNA template
	1 cycle:	
	72 °C - 5 min	

Table 3. Distribution of UPEC strains in diabetic patients and prevalence of antibiotic resistance patterns

No. urine samples of diabetic patients	Positive results (%)	Antibiotic resistance patterns (%)											
		AM10*	TE30	GM10	AMK 30	IMP30	MEZ30	PIP30	CIP5	Norf30	Cotr30	CFTZ30	OFLX5	CF30
110	33 (30)	28 (84.8)	29 (87.8)	28 (84.8)	20 (60.6)	1 (3.0)	8 (24.2)	13 (39.3)	25 (75.7)	15 (45.4)	17 (51.5)	11 (33.3)	14 (42.4)	18 (54.5)

*In this table: AM10= ampicillin (10 u/disk); TE30= tetracycline (30 µg/disk); GM10= gentamycin (10 µg/disk); AMK30= amikacin (30 u/disk); IMP30= imipenem (30 u/disk); MEZ30= mezlocillin (30 u/disk); pip30= piperacillin (30 µg/disk); CIP5= ciprofloxacin (5 µg/disk); norf30= norfloxacin (30 µg/disk); cotr30= cotrimoxazole (30 µg/disk); CFTZ30= ceftazidime (30 µg/disk); OFLX5= ofloxacin (5 µg/disk); CF30= cephalothin (30 µg/disk).

Table 4. Distribution of Uropathogenic virulence genes in diabetic patients of Iran

No. urine samples of diabetic patients	Positive results (%)	Virulence genes (%)										
		set-1	*astA*	*sigA*	*PapGI*	*PapGII*	*PapGIII*	*fim*	*Iha*	*usp*	*afa*	*sfa*
110	33 (30)	8 (24.2)	5 (15.1)	2 (6.0)	10 (30.3)	10 (30.3)	9 (27.2)	15 (45.4)	2 (6.0)	1 (3.0)	12 (36.3)	15 (45.4)

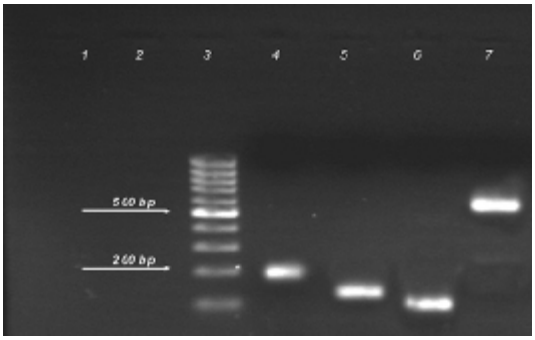

Fig. 1. Gel Electrophoresis of PCR products. 3: 100 bp ladder and 4-7: positive samples for *papGII*, *set-1*, *astA* and *fim*, respectively

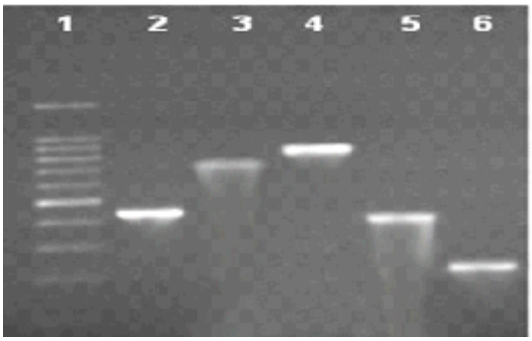

Fig. 2. Gel Electrophoresis of PCR products. 1: 100 bp ladder and 2-6: positive samples for *papGI*, *afa*, *E. coli 16S rRNA*, *papGIII*, *sigA* and *papGII*, respectively.

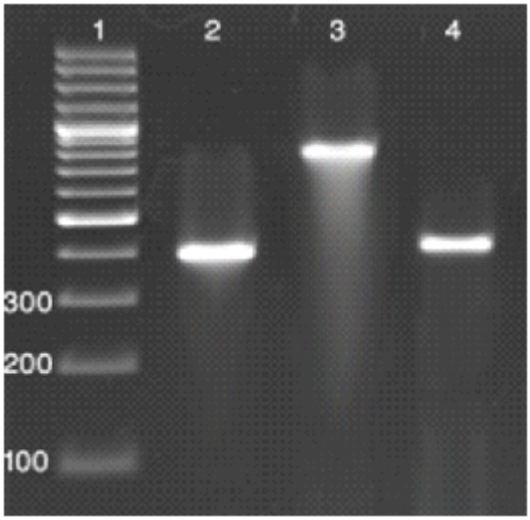

Fig. 3. Gel Electrophoresis of PCR products. 1: 100 bp ladder and 2-4: positive samples for *afa*, *iha* and *usp*, respectively

sfa and *iha* (P =0.011), *fim* and *usp* (P =0.021) and also between *afa* and *sigA* genes (P =0.031).

DISCUSSION

The results of our investigation showed that UPEC strains had the high prevalence in the urine samples of diabetic patients suffered from UTIs. Similar results have been reported previously by Naveen and Mathai (2005) (11), Ahmed *et al*. (2014) (12) and Boyko *et al*. (2002) (13). However, Ghengesh *et al* (2009) (5) found that there was a trend towards a lower proportion of UTIs caused by this organism in DM compared with non-DM patients (13 vs 18 %, respectively). Although our findings are similar to those of Bonadio *et al*. (1999) (14) and Ghengesh *et al* (2009) (5) as far as *E. coli* is important concern in diabetic patients.

Several studies have been performed on the prevalence of UPEC strains in cases of UTIs in Iran (1, 15, 16). Kalantar *et al*. (2008) (15) showed that the *E. coli* was the most frequently pathogen (54.80%) in patients with UTIs. Esmaeili (2005) (16) showed that the *E. coli* was the most common cause of UTI in Iranian people. Nazemi *et al*. (2011) (17) reported that of 244 urine samples studied, 140 samples (57.37%) were infected with UPEC strains which was entirely higher than our results. Several reports have been performed on the prevalence of UPEC strains around the world including United States (18), Brazil (19) and Europe (20).

The high differences in prevalence of UPEC strains in the urine samples maybe due the facts that type of samples (DM or non DM, women or men, young or old and …), number of samples, methods of sampling, method of experiment, geographical area and even climate of area which samples were collected are different in each investigation.

Antimicrobial resistance among UPEC strains causing hospital-acquired and community-UTIs is increasing (20). Few data are available on the role of DM as a risk factor for the development of antimicrobial resistance in the UPEC strains. Bonadio *et al*. (2001) reported that the resistance of uropathogens to antibiotics was similar in patients with and without DM (20). Although, Ghengesh *et al*. (2009) (5) found no differences in the resistance profiles of UPEC strains regardless

of whether they were isolated from DM or non-DM patients with UTIs.

E. coli strains of our study were resistant to all of the tested antibiotics. In fact, most of strains were resistant to tetracycline (87.8%), ampicillin (84.8%), gentamycin (84.8%), ciprofloxacin (75.7%) and cephalothin (54.5%) but some of them were sensitive to imipenem (3%) and mezlocillin (24.2%). Similar results have been reported by Ghengesh *et al*. (2009)[5], Momtaz *et al*. (2012)[1] and Dormanesh *et al*. (2014)[6]. Exorbitant and high irregular medical prescription of cephalothin, trimethoprim, tetracycline, ampicillin and ciprofloxacin, antibiotics caused to UPEC strains of our study were absolutely resistant to these antibiotics.

Many virulence genes have been reported in UPEC and are frequently associated with UTIs[1]. Studies have shown that hosts with predisposing factors, such as DM, can acquire UTIs caused by less virulent *E. coli* strains[21]. Besides, Ghengesh *et al*. (2009)[5] showed similar findings with those of Tseng *et al*. (2002)[21], with multivirulent UPEC isolates being detected significantly more in non-DM than in DM patients. Despite the results of Tseng *et al*. (2002) (21) and Ghengesh *et al*. (2009)[5], our results showed that virulent strains of uropathogenic *E. coli* are responsible for causing UTIS in diabetic patients of Iran. Totally, *sfa* (45.4%), *fim* (45.4%) and *afa* (36.3%) were the most commonly detected virulence factors. It seems that several of the virulence genes detected in our investigation, such as *set-1*, *astA*, *sigA* and *iha*, have not been reported previously in the UPEC strains isolated from diabetic patients.

In conclusion, we identified a large number of virulence factors and antibiotic resistance properties in the UPEC strains of diabetic patients of Iran. *E. coli* causing UTI in diabetic patient populations differ in their pathogenic potential and susceptibility to antimicrobial agents. This has to be taken into account while developing guidelines for management of UTI. Also, judicious use of antibiotics is required by clinicians.

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