Distribution of Virulence Factors and Antimicrobial Resistance Properties of Uropathogenic *Escherichia coli* Isolated from Diabetic and Healthy Males Suffered from Urinary Tract Infections

Reza Rezaee[1], Amir Talebreza[2], Katayoun Ziari[3], Vahid Behnod[4] and Bibi Fatemeh Shakhs Emampour[5]*

[1]Ministry of Health and Medical Education,
Deputy of Curative Affaires Budget Administration , Tehran , Iran.
[2]Department of Surgery , AJA University of Medical Sciences, Tehran, Iran.
[3]Department of Pathology, Be'sat Hospital, AJA University of Medical Sciences, Tehran, Iran.
[4]Baqiyatallah University of Medical Sciences, Tehran, Iran.
[5]Department of Anesthesiology , Birjand University of Medical Sciences, Birjand, Iran.

Urinary tract infections and diabetes are two of the most important infectious and metabolic diseases all-around the world. Documented data showed that diabetes is one of the most important causative reasons of getting UTIs. Virulent and resistant strains of *Escherichia coli* are the most important causative agents for UTIs. The present investigation was carried to in order to evaluate the distribution of uropathogenic *E. coli* as well as virulence factors and antimicrobial resistance pattern of bacterial isolates of diabetic and non-diabetic patients suffered from UTIs. A total of 300 urine samples were collected from diabetic and non-diabetic patients suffered from UTIs. Samples were cultured and those that were positive were subjected to PCR and disk diffusion method. Prevalence of UPEC strains in diabetic and healthy patients were 65.38% and 36.47%, respectively. Significant difference was seen for the prevalence of UPEC strains between diabetic and healthy male patients (P <0.01). Older patients had the higher prevalence of *E. coli* (P <0.01). *Fim*, *cnf1*, *papGIII* and *hlyA* were the most commonly detected virulence factors. UPEC strains showed the highest levels of resistance against ampicillin, gentamicin, ciprofloxacin and trimethoprim-sulfamethoxazole. UPEC strains of diabetic patients were more virulent and resistant (P <0.05). Prescription of ceftriaxone, aztreonam, nalidixic acid and imipenem can reduce the risk of UTIs in diabetic patients.

Key words: Uropathogenic *Escherichia coli*, Virulence factors, Antibiotic resistance, Diabetes.

Diabetes and urinary tract infections (UTIs) are two most prominent diseases all-around[1]. Diabetes is associated with an earlier onset and increased severity of UTIs, resulting in costly and debilitating complications. Several investigations showed the higher distribution of UTIs, urethritis, pyelonephritis, cystitis and bladder dysfunction in diabetic in compare with non diabetic patients[1-3].

Documented data revealed that near to 50% of people have been affected by UTIs all-around the world[4]. Uropathogenic *Escherichia coli* (*E. coli* (UPEC)) strains are the most com-mon causes of UTIs[5, 6]. Evaluating the potential virulence genes is required to assess the pathogenicity of UPEC strains in UTIs. Successful colonization, establishment, and ultimately leading to UTIs by UPEC strains is based on the ability to

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* To whom all correspondence should be addressed.
Tel.: 00989155144073;
E-mail: f.emampour@gmail.com

adhere to host surfaces such as mucous membranes, urinary epithelial or kidney tissue. The most important virulence factors in UPEC strains are hemolysin (*hly*), P fim-briae (*pap*cytotoxic necrotizing factor 1 (*cnf 1*),), a fimbrial adhesin (*afa*), and S fimbriae (*sfa*)[5-8]. Successful colonization and invasion of UPEC in the urinary tract depends on the expression of mentioned virulence factors.

One of the most essential aspects for control of the UTIs is treatment. Treatment of diseases caused by this bacterium often requires antimicrobial therapy; however, antibiotic-resistant strains of *E. coli* bacterium cause more severe diseases for longer periods of time than their susceptible isolates. Several studies showed that antibiotic resistance in UPEC is increasing nowadays[8-10].

Iran is one of the most important sites of the world which has a high prevalence of UTIs caused by UPEC strains and also diabetes[8, 11-13]. Therefore, epidemiological researches should be done to find the exact pathogenicity and antimicrobial resistance properties of various strains of *E. coli* in the cases of UTIs. The current survey was carried out in order to determine the distribution of virulence factors and antimicrobial resistance properties of UPEC strains isolated from diabetic and non diabetic patients suffered from UTIs.

MATERIALS AND METHODS

Samples and *Escherichia coli* identification

This cross sectional study was performed from May to December 2015. A total of 300 urine samples were collected from male patients with UTIs. In the other hand, urine samples were collected from diabetic (n= 130) and healthy (n= 170) male patients suffered from UTIs. Patients of our study had the various ranges of age including 20-30, 30-40, 40-50, 50-60 and older than 60 years old. All samples were collected from the hospitalized pediatrics of educational hospitals in Tehran, Iran. Midstream urine was collected in sterile condition to decrease potential bacterial, cellular and artifactual contamination. All samples were immediately transferred to the laboratory at 4°C. 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 24h. One milliliter sample of the nutrient broth culture was mixed with 9 mL of MacConkey broth (Merck, Germany) and further incubated at 37 °C for 24h. 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) plate (Merck, Germany) and incubated at 37 °C for 24h. A metallic green colony from each plate with typical *E. coli* morphology was selected and examined by biochemical tests, including hydrogen sulfide, citrate, urease and indole.

Antimicrobial Susceptibility Testing

The antibiotic susceptibility patterns were determined using the disk diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (20). The following antimicrobials were tested: cefoxitin (FOX: 30 ìg), amoxicillin-clavulanic acid (AMC: 20/10 µg), ceftriaxone (CRO: 30 ìg), gentamicin (GEN: 10 ìg), ampicillin (AMP: 10 ìg), nitrofurantoin (NIT, 300 µg), ceftazidim (CAZ: 30 ìg), aztreonam (ATM: 30 ìg), nalidixic acid (NA: 30µg), imipenem (IMP: 10 ìg), ciprofloxacin (CIP: 5 ìg) and trimethoprim-sulfamethoxazole (SXT: 25 ìg). The quality control organism was *E. coli* ATCC 25922. Results were interpreted as susceptible or resistant according to criteria recommended by the CLSI and the manufacture protocols (Mast Companies, UK) (14).

DNA extraction and *E. coli* identification

Bacterial strains were sub cultured overnight in Luria Bertani broth (Merck, Germany) and genomic DNA was extracted from typical colonies of *E. coli* using DNA extraction kit (Fermentas, Germany) according to manufacturer's instruction. All of the positive colonies were confirmed using the polymerase chain reaction (PCR) technique (15). PCR was performed with a total volume of 50 µL including 2 mM MgCl2, 1 µM of forward primer (5'-AGAGTTTGATC MTGGCTCAG-3'), 1 µM of reverse primer (5'-CCGTCAATTCATTTGAGTTT-3'), 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 45s, primer annealing at 59°C for 60s, and DNA chain extension at 72°C for 60s.

Amplification of virulence factors

E. coli strains were cultured in LB broth

at 37°C for 18 hours. Genomic DNA was extracted from the bacterial colonies using the DNA extraction kit (Fermentas, Germany) according to the manufacture's instruction. Table 1 shows the list of primers used for amplification of latent virulence factors[8]. All of the PCR reactions were done using the programmable thermocycler (Mastercycler Gradiant Eppendorph, Germany). A PCR method was performed with a total volume of 50 µL including 1.5 mM MgCl2, 0.4 µM of forward primer, 0.4 µM of reverse primer, 5 µL PCR buffer 10X, 200 µM dNTP (Fermentas), 1 U Taq DNA polymerase (Fermentas), and 4 µL DNA template. The DNA was then amplified by 30 suc cessive cycles of denaturation at 94°C for 60s, primer annealing at 63°C for 30s, and DNA chain extension at 72°C for 90s with a programmable thermal cycler (Eppendorf, Mastercycler® 5330, Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany). *E. coli* ATCC 25922 and sterile distilled water were used as positive and negative controls in all PCR reactions.

Gel electrophoresis

All 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.

Statistical analysis

Data were analyzed using SPSS software (Version 17. SPSS Inc, United States) to find any significant correlation between incidences of virulence factors and antibiotics resistance pattern of uropathogenic *E. coli* isolated from diabetic and healthy males with urinary tract infection. Statistical significance was regarded at a P value < 0.05.

RESULTS

Results of the present investigation showed that *E. coli* strains had a high prevalence in diabetic and non diabetic patients suffered from UTIs. Table 2 represents the total distribution of Uropathogenic *E.coli* in diabetic and healthy patients of various age groups suffered from UTIs. We found that the total prevalence of *E. coli* in diabetic and healthy patients were 65.38% and 36.47%, respectively. Significant statistical difference was seen for the prevalence of *E. coli* between diabetic and healthy male patients (P <0.01).The most commonly infected groups were older than 60 and 50-60 years old patients.

Table 1. List of primers used for amplification of latent virulence factors in the *Escherichia coli* strains of diabetic and healthy males suffered from urinary tract infections

Target gene	Primer sequence (5'-3')	Size of product (bp)
papGI	TCGTGCTGAGGTCCGGAATTT TGGCATCCCCCAACATTATCG	461
papGII	GGGATGAGCGGGCCTTTGAT CGGGCCCCCAAGTAACTCG	190
papGIII	GGCCTGCAATGGATTTACCTGG CCACCAAATGACCATGCCAGAC	258
fim	GAGAAGAGGTTTGATTTAACTTATTG AGAGCCGCTGTAGAACTGAGG	559
afa	GCTGGGCAGCAAACTGATAACTCTC CATCAAGCTGTTTGTTCGTCCGCCG	750
sfa	CTCCGGAGAACTGGGTGCATCTTAC CGGAGGAGTAATTACAAACCTGGCA	410
hlyA	AACAAGGATAAGCACTGTTCTGGCT ACCATATAAGCGGTCATTCCCGTCA	1177
cnf1	AAGATGGAGTTTCCTATGCAGGAG TGGAGTTTCCTATGCAGGAG	498

Table 2. Total distribution of Uropathogenic *Escherichia coli* in diabetic
and healthy patients of various age groups suffered from UTIs

Types of samples		No. samples	*E. coli* positive by culture (%)	PCR confirmation (%)
Diabetic patients	20-30	21	7 (33.33)	7 (33.33)
	30-40	23	10 (43.47)	10 (43.47)
	40-50	20	12 (60)	12 (60)
	50-60	22	16 (72.72)	16 (72.72)
	60 <	46	40 (86.95)	40 (86.95)
	Total	130	85 (65.38)	85 (65.38)
Healthy patients	20-30	20	4 (20)	4 (20)
	30-40	28	9 (32.14)	9 (32.14)
	40-50	32	11 (34.37)	11 (34.37)
	50-60	37	14 (37.83)	14 (37.83)
	60 <	53	24 (45.28)	24 (45.28)
	Total	170	62 (36.47)	62 (36.47)

Table 3. Total distribution of Uropathogenic virulence factors in
the *Escherichia coli* strains of diabetic and healthy patients suffered from UTIs

Types of samples (No. positive)	Distribution of virulence factors (%)							
	papGI	*papGII*	*papGIII*	*fim*	*cnf1*	*afa*	*sfa*	*hlyA*
Diabetic patients (85)	13 (15.29)	17 (20)	80 (94.11)	82 (96.47)	80 (94.11)	74 (87.05)	78 (91.76)	79 (92.94)
Healthy patents (62)	9 (14.51)	13 (20.96)	57 (91.93)	58 (93.54)	57 (91.93)	49 (79.03)	54 (87.09)	57 (91.93)
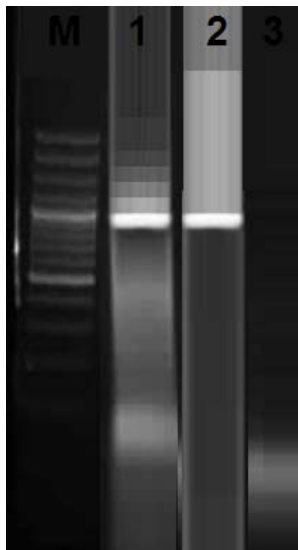
Fig. 1. Results of the gel electrophoresis for confirmation of Escherichia coli in the genomic DNA extracted from the bacterial colonies. M: 100 bp ladder, 1: positive samples for the *16SrRNA* gene (919 bp), 2: Positive control and 3: negative control

Statistically significant difference was seen for the prevalence of *E. coli* between various age groups (P <0.01).

Table 3 shows the total distribution of Uropathogenic virulence factors in the *E. coli* strains of diabetic and healthy patients suffered from UTIs. The most commonly detected virulence factors in the UPEC strains of the diabetic patients were *fim* (96.47%), *cnf1* (94.11%), *papGIII* (94.11%) and *hlyA* (92.94%). Those of non-diabetic patients were *fim* (93.54%), *hlyA* (91.93%) and *cnf1* (91.93%). Statistically significant differences were seen between the prevalence of various virulence factors (P <0.05).

Table 4 represents the antibiotic resistance pattern of Uropathogenic *E. coli* strains of diabetic and healthy patients suffered from UTIs. *E. coli* strains harbored the highest levels of resistance against ampicillin, gentamicin, ciprofloxacin and trimethoprim-sulfamethoxazole. This finding in the diabetic patients was higher than healthy ones but there were no significant differences.

Table 4. Antibiotic resistance pattern of Uropathogenic *Escherichia coli* strains of diabetic and healthy patients suffered from UTIs

Types of samples (No. positive)	Antibiotic resistance pattern (%)											
	FOX	AMC	CRO	GEN	AMP	NIT	CAZ	ATM	NA	IMP	SXT	CIP
Diabetic patients (85)	23 (27.05)	55 (64.70)	19 (22.35)	83 (97.64)	84 (98.82)	25 (29.41)	26 (30.58)	13 (15.29)	16 (18.82)	5 (5.88)	61 (71.76)	70 (82.35)
Healthy patents (62)	15 (20.96)	40 (64.51)	11 (17.74)	58 (93.54)	59 (95.16)	19 (30.64)	21 (33.87)	7 (11.29)	9 (14.51)	1 (1.61)	48 (77.41)	59 (95.16)

DISCUSSION

The results of te present investigation revealed that diabetic patients and especially diabetic males are more prone to get UTIs tan non diabetic patients. Total prevalence of UPEC strains in diabetic and non diabetic patients were 65.38% and 36.47%, respectively. Higer prevalence of resistant and virulent strains of *E. coli* in diabetic patients was another important finding of our study.

Higher prevalence of virulent and resistant UPEC strains in diabetic than non diabetic patients is due to the fact that diabetes is a causative agent for the suppression of level of immunity in human. In this situation, occurrence of infections like UPEC strains has been increased. The mechanisms of diabetes caused them to more sensitive to various types of infections. Decrease in the level of immunity caused to occurrence of higher resistant of bacterial strains against commonly used antibiotics. Besides, lack of powerful immunity caused bacterial strains to produce more important secretary and non secretary virulence factors.

To our best knowledge the frequency of epidemiological investigations in this field is so scarce. In a study which was conducted in order to investigate the prevalence of virulence factors and phylogenetic characterization of uropathogenic *E. coli* causing urinary tract infection in patients with and without diabetes mellitus[16], results showed that there was no significant difference in distribution of virulence factors of UPEC causing UTI from patients with and without diabetes. *PapC* gene was most prevalent in both groups of patients, followed by *hly* gene which was similar to our findings. Only *cnf-1* gene was observed to be significantly associated (p<0.05) with the non-diabetic status than diabetic. Bangal investigation[17] showed that *E. coli* was the most prevalent cause of UTIs in diabetic patients. Amikacin exhibited only 3% resistance and gentamicin exhibited 26.9% resistance with *E. coli*. *Prevalence of resistance against* nitrofurantoin was low. Besides, resistance against cefixime and ceftriaxone was moderate and amoxicillin and ciprofloxacin showed the highest resistances in all these cases which wall were similar to our results. In a study which was conducted by Hamdan et al. (2015)[18], the predominant isolates

were *E. coli* (56.4%). Six, four, three, and two of 22 *E. coli* isolates showed resistance to ampicillin, co-trimoxazole, nitrofurantoin, and amoxicillin-clavulanic acid, respectively. In addition, all 22 *E. coli* isolates were sensitive (100%) to gentamicin and cephalexin.

Our result represents that the prevalence of UPEC resistance against amoxicillin-clavulanic acid gentamicin, ampicillin, ciprofloxacin and trimethoprim-sulfamethoxazole were more than 60% which was considerable high. Irregular and excessive administration of antibiotics is the most important reason for the high prevalence of resistance against commonly used antimicrobial agents in our study. In fact, Medical practitioners don't use from rapid and simples methods like disk diffusion technique to evaluate the exact profile of antibiotic resistance in the cases of UTIs especially those caused by UPEC strains. Therefore, antibiotic resistance will occur in a short period of time. Differences in the levels of antibiotic resistance which were showed in various studies maybe due to the differences in the availability of antibiotics, pattern of resistance, idea of medical practitioners to antibiotic administration and even cost of antibiotic agents in each zone and/or country.

We also found that the UPE3C strains harbored the high levels of virulence factors and especially *fim*, *cnf1*, *papGIII* and *hlyA*. Momtaz et al. (2013)[8] represented that *set1*, *fim*, *cnf1*, *papGIII* and *hlyA* were the most commonly detected virulence factors in the UPEC strains of patients suffered from UTIs which was entirely similar to our findings. High prevalence of *fim*, *hly*, *sfa*, *afa* and also various types of *pap* genes were also reported by Dormanesh et al. (2014)[15], Arabi et al. (2012)[19], Asadi Karam et al. (2012)[20], Karimian et al. (2012)[21], Harwalkar et al. (2013)[22], Yun et al. (2014)[23] and Zhao et al. (2009)[24]. These genes are mainly associated with adhesion, colonization and invasion of bacterial strains into the urinary epithelial cells.

CONCLUSIONS

In conclusions, we identified a large numbers of virulence factors and antimicrobial resistance properties in the UPEC strains isolated from diabetic and non diabetic patients suffered from UTIs. Higher numbers of UPEC strains, virulence factors and also antibiotic resistance pattern in the diabetic than non-diabetic patients are other important findings of our investigation. Older patients due to their lower levels of immunity had the highest prevalence of UPEC strains. The most commonly detected virulence genes are *fim*, *cnf1*, *papGIII* and *hlyA* and UPEC strains harbored the highest levels of resistance against amoxicillin-clavulanic acid gentamicin, ampicillin, ciprofloxacin and trimethoprim-sulfamethoxazole antimicrobial agents. We found that prescription of ceftriaxone, aztreonam, nalidixic acid and imipenem regarding the results of the disk diffusion can reduce the risk of UTIs in diabetic patients.

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