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Virulence Factor Diversity Between Imipenem Resistant and Imipenem Susceptible Strains of *Escherichia coli* Isolated from Hospitalized Patients with Severe Urinary Tract Infections

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From a clinical perspective, it is imperative to discern the differences in the distribution of virulence factors between imipenem resistant and imipenem susceptible strains of uropathogenic *Escherichia coli* isolated from hospitalized patients. The present study was carried out to find this purpose. One-hundred and sixty urine specimens of children and seniors were collected from the Educational Hospital of Tehran, Iran. The urine samples were cultured immediately and those that were *E. coli*-positive were analyzed for the antimicrobial susceptibility pattern against imipenem using disk diffusion method. Imipenem resistant and imipenem susceptible strains were analyzed for the presence of *sfa*, *afa*, *pap*, *hly*, *cnf1* and *fim* virulence factors using the PCR technique. Fifty out of 70 (71.42%) children urines and 55 out of 90 (61.11%) seniors urine samples harbored *E. coli*. The incidence of resistance against imipenem in children and seniors were 6% and 9.09%, respectively. In the other hand, 99.39% of tested strains were susceptible to imipenem. Total prevalence of *pap*, *cnf1*, *hly*, *sfa*, *afa* and *fim* genes in the imipenem resistant strains were 12.5% 25%, 50%, 75%, 62.5% and 25%, respectively. Prevalence of the *sfa, afa* and *hly* genes was lower in the imipenem susceptible strains of *E. coli*, while the *pap*, *cnf1* and *fim* genes was entirely higher in the imipenem susceptible strains. This finding suggests that imipenem resistance could be directly associated with decreased prevalence of *pap*, *cnf1* and *fim* virulent genes. However, the findings of the present study are novel and valuable but more courtesy studies are necessary to authorize them.

Key words: Urpathogenic *Escherichia coli*, Virulence factors, Imipenem resistant, Hospitalized patients, Urinary tract infections.

Urinary Tract Infections (UTIs) are common bacterial infectious diseases causing illness in majority of people. The Uropathogenic *Escherichia coli* (*E. coli*) (UPEC) is one of the main etiologic agents for UTIs. It has been appraised that the UPEC strains are responsible for 70-90% of cases of UTIs in children and 40-50% of UTIs cases in seniors (Schalger 2001; Riccabona 2003; Rajan and Prabavathy, 2012). Based on the report of World Health Organization (WHO), UTIs are determined as a common cause of UTIs in 1% of boys and 3–8% of girls (WHO 2005; Foxman 2003).

Treatment is a critical point in the epidemiology of UTIs in children and seniors, while therapeutic options have become somewhat limited because of the presence of multi drug resistant strains of UPEC. Imipenem is sometimes the only effective agent for treatment of severe UTIs caused by multi drug resistant UPEC (Hong *et al*., 2005).

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Farshad *et al*. (2012), Sharmin *et al*. (2009) and Ponnusamy *et al*. (2012) showed that all strains of *E. coli* isolated from UTIs were sensitive to the Imipenem. In *E. coli*, reports of low-level resistance to imipenem are extraordinary, resulting from AmpC hyperproduction and loss of porins (Low *et al*., 2001; Nordmann and Poirel, 2002).

To appraise the pathogenicity of UPEC strains in UTIs, assessment of latent virulence factors is requisite. A compulsory state in the successful immigration, establishment, and ultimately production of infection by UPEC strains is an aptitude to adhere to host surfaces such as mucous membranes, urinary epithelial or kidney tissue. The virulence genes that are most commonly associated with UPEC include P fimbriae (*pap*), aerobactin (*aer*), hemolysin (*hly*), a fimbrial adhesin I (*afa*I), type 1 fimbria (*fim H*), cytotoxic necrotizing factor 1 (*cnf* 1) and S fimbriae (*sfa*) (Momtaz *et al*., 2013; Soto *et al*., 2009).

The *sfa* gene is responsible for adhesion to the endothelial and epithelial cells of the lower urinary tract and kidney tissues (Mulvey 2002). The *cnf1* gene is produced by 1/3 of all pyelonephritis strains and may also be involved in kidney damages, bladder cell exfoliation and enhancement of bacterial access to underlying tissue (Mills *et al*., 2000). The *hly* gene is associated with upper UTIs such as pyelonephritis and also is able to lyses nucleated host cell, damage effectors' immune cells, induce the apoptosis of T lymphocytes, neutrophils and renal cells, and gain enhanced access to host nutrients and iron stores (Bien *et al*., 2012; Los *et al*., 2013). Clinical findings recommend that *UPEC strains* with *afa* adhesins have characters potentially favoring the occurrence of pyelonephritis, recurrent and chronic UTIs (Le Bouguénec 2005).

There were several published investigations showed that resistant strains of *E. coli* are less able to cause upper urinary tract infection and have fewer virulence factors than susceptible strains (Vila *et al*., 2002; Farshad *et al*., 2010). Some in vitro studies have also suggested that decreased pathogenicity of *E. coli* is associated with the acquisition of antibiotic resistance (Vila *et al*., 2002; Piatti *et al*., 2008; Jadhav *et al*., 2011). Resistant strains of UPEC harbored prosperous and diverse amount of virulence factors than susceptible strains (Piatti *et al*., 2008; Jadhav *et al*., 2011). Therefore, it is important to know the differences of virulence factors profile between resistant and susceptible strains of UPEC. With respect to the high importance of imipenem as a best choice for treatment of UTIs in Iran, the present study was carried out in order to investigate the distribution of virulence factors among imipenem resistant and imipenem susceptible strains of UPEC isolated from hospitalized patients with severe urinary tract infections in Iran.

MATERIALS AND METHODS

Samples collection

From March to December 2012, a total of 160 urine samples were collected from children (n=70) and seniors (n=90) hospitalized patients who suffered from UTIs. Presence of UTIs in pediatrics was confirmed using the ultrasound technique (MacKenzie *et al*., 1994). All samples were collected from the hospitalized pediatrics of Educational Hospital in Tehran, Iran. Most of patients had been handling urine catheter for a week before they got UTIs. Strong urge to urine frequently even immediately after the bladder is emptied, painful burning sensation when urinating, cloudy and bloody urine with bad smell and in some cases fever, chills and nausea are the most commonly detected symptoms in patients. In order to decrease potential bacterial, and cellular contamination, all urine samples were collected from midstream. Urine samples were collected using the Supra pubic Aspiration (SPA) method based on the standard (NICE 2007).

Escherichia coli isolation

All samples were immediately transferred to the Microbiology Research center of the Islamic Azad University 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 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 *E. coli* strains against imipenem antibiotic was determined using the instruction of Clinical and Laboratory Standards Institute guidelines (CLSI 2012). Susceptibility of *E. coli* isolates were tested against imipenem (30 u/disk) antibiotic agent (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). In all reactions, the *E. coli* ATCC 25922 was used as quality control organisms.

DNA extraction and Polymerase Chain Reaction (PCR)

Resistant and susceptible strains of *E. coli* were cultured overnight on Luria-Bertani broth (Merck, Germany) and genomic DNA was extracted from typical colonies using the DNA extraction kit (Fermentase, Germany) according to manufacturer's instruction. Resistant and susceptible strains of *E. coli* were also confirmed using the PCR technique (14). 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. The programmable thermal cycler (Eppendorf, Flexercycler^2® 5330, Eppendorf-Netheler-Hinz GmbH, Hamburg,Germany) PCR device was used in all PCR reaction.

Detection of UPEC virulence factors

Several PCR reactions were used for detection of virulence factors in imipenem resistant and imipenem susceptible strains of *E. coli*. List of primers used for detection of virulence genes is shown in Table 1. PCR conditions are shown in Table 2.

PCR products were electrophoresed using 2% agarose gels which was stained with ethidium bromide at 90 V for 6 h using 1× TBE (0.89 M Tris borate, 0.02 M EDTA, pH 8.3) as the running buffer. All products were examined under ultraviolet illumination. A set of molecular weight standards (Fermentas, GmbH, Germany) ranging from 100 bp to 2000 bp was included on each gel. In order to confirm the PCR results, the sequencing method was used. For this reason, PCR products of some positive samples were purified with High pure PCR product purification kit (Roche Applied Science, Germany) according to manufacturer's recommendations. Single DNA strands were sequenced with ABI 3730 XL device and Sanger sequencing method (Macrogen, Korea). Result of the sequence of each gene was aligned with the gene sequences recorded in the GenBank database located at NCBI.

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 for the distribution of virulence genes between the resistant and susceptible strains of *E. coli* isolated from hospitalized patients with UTIs. A P value < 0.05 was considered statistically significant.

Ethical issues

The present study was authorized by the ethical committee of the Educational Hospital of Tehran, Iran, and the Microbiology and Infectious Diseases Center of the Islamic Azad University of Shahrekord Branch, Iran. All patients or their parents signed the written informed consent.

RESULTS

All of the urine samples of hospitalized children and seniors were examined for *E. coli* using culture and PCR techniques (Figure 1 and 2). From 160 urine samples, 105 (65.62%) were positive for *E. coli* (Table 3). In addition, 55 out of 90 urine samples of seniors (68.75%) and 50 out of 70 urine samples of children (36.90%) were positive for *E. coli*. There were no significant

differences in the incidence of *E. coli* between children and seniors.

Of 105 isolates of *E. coli*, 8 strains were imipenem resistant (7.61%) (Table 3). We found statistically significant (P =0.012) association between the incidence of imipenem resistant and imipenem susceptible strains of *E. coli*.

Distribution of putative virulence factors among imipenem resistant and imipenem susceptible strains of *E. coli* is shown in table 4. Some different profiles of virulence factors have been seen in the imipenem resistant and imipenem susceptible strains of *E. coli*. The most commonly detected virulence factors in imipenem resistant

Table 1. Oligonucleotide primers for detection of various putative virulence genes of Uropathogenic *Escherichia coli* isolated from hospitalized patients

Gene	Primer name	Primer Sequence (5'-3')	Size of product (bp)	Reference
pap	pap3	GCAACAGCAACGCTGGTTGCATCAT	336	Yamamoto
	pap4	AGAGAGAGCCACTCTTATACGGACA		*et al*., 1995
cnf1	cnf1	AAGATGGAGTTTCCTATGCAGGAG	498	Yamamoto
	cnf2	TGGAGTTTCCTATGCAGGAG		*et al*., 1995
hly	hly1	AACAAGGATAAGCACTGTTCTGGCT	1177	Yamamoto
	hly2	ACCATATAAGCGGTCATTCCCGTCA		*et al.,* 1995
sfa	sfa1	CTCCGGAGAACTGGGTGCATCTTAC	410	Le Bouguenec
	sfa2	CGGAGGAGTAATTACAAACCTGGCA		*et al*., 1992
afa	afa1	GCTGGGCAGCAAACTGATAACTCTC	750	Le Bouguenec
	afa2	CATCAAGCTGTTTGTTCGTCCGCCG		*et al*., 1992
fim	fim1	GAGAAGAGGTTTGATTTAACTTATTG	559	Struve *et al*.,
	fim2	AGAGCCGCTGTAGAACTGAGG		1999

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

Gene	PCR program	PCR volume (50 µL)
16srRNA	1 cycle:	5 µL PCR buffer 10X
	95 °C -6 min.	2 mM $Mgcl_2$
	31 cycle:	200 µM dNTP (Fermentas)
	95 °C -45 s	1 µM of each primers F & R
	59 °C -60 s	1 U Taq DNA polymerase (Fermentas)
	72 °C -60 s	2.5 µL DNA template
	1 cycle:72 ^{0C} -5 min	
fim	1 cycle:	5 µL PCR buffer 10X
	94 °C -3 min.	1.25 mM $Mgcl_2$
	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	
pap, sfa, afa,	1 cycle:	5 µL PCR buffer 10X
	94 °C -1 min.	1.5 mM $Mgcl_2$
	30 cycle:	200 µM dNTP (Fermentas)
hlyA, cnf1	94 °C -60 s	0.4 µM of each primers F & R
	63 °C -30 s	1 U Taq DNA polymerase (Fermentas)
	72 °C -90 s	4 µL DNA template
	1 cycle:72 ^{0C} -5 min	

Table 3. Distribution of imipenem resistant and imipenem susceptible strains of Uropathogenic *Escherichia coli* isolated from hospitalized patients

Type of urine samples	No. samples	No. positive samples (%)	No. Imipenem resistant (%*)	No. Imipenem susceptible (%*)
Seniors	90	55 (61.11)	5 (9.09)	50 (90.91)
Children	70	50 (71.42)	3 (6)	47 (94)
Total	160	105 (65.62)	8 (7.61)	97 (92.39)

*Percentages are based on the positive samples

Table 4. Distribution of putative virulence factors among imipenem resistant and imipenem susceptible strains of Uropathogenic *Escherichia coli* isolated from hospitalized patients

No. positive samples		No. positive samples	Virulence factors (%)					
			pap	*cnf1*	*hly*	*sfa*	*afa*	*fim*
Seniors	Imipenem resistant	5	1 (20)	1 (20)	3 (60)	4 (80)	3 (60)	2 (40)
	Imipenem susceptible	50	23 (46)	27 (54)	20 (40)	25 (50)	15 (30)	20 (40)
Children	Imipenem resistant	3	-	1 (33.33)	1 (33.33)	2 (66.66)	2 (66.66)	-
	Imipenem susceptible	47	17 (36.17)	22 (46.80)	12 (25.53)	22 (46.80)	20 (42.55)	19 (40.42)
Total	Imipenem resistant	8	1 (12.5)	2 (25)	4 (50)	6 (75)	5 (62.5)	2 (25)
	Imipenem susceptible	97	41 (42.26)	50 (51.54)	15 (32.98)	47 (48.45)	35 (36.08)	39 (40.20)

strains of *E. coli* were *sfa* (75%), *afa* (62.5%) and *hly* (50%), while *cnf1* (51.54%), *pap* (42.26%) and *fim* (40.20%) were the most commonly detected virulence factors in imipenem susceptible strains of *E. coli* (Table 4).

There were significant differences (P =0.031) in the incidence of *sfa* gene between the imipenem resistant and imipenem susceptible

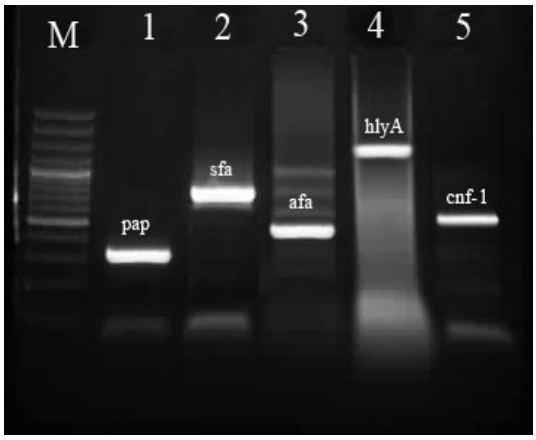

Fig. 1. Gel electrophoresis of PCR products for Uropathogenic virulence factors. M: 100bp ladder and 1-5: positive samples

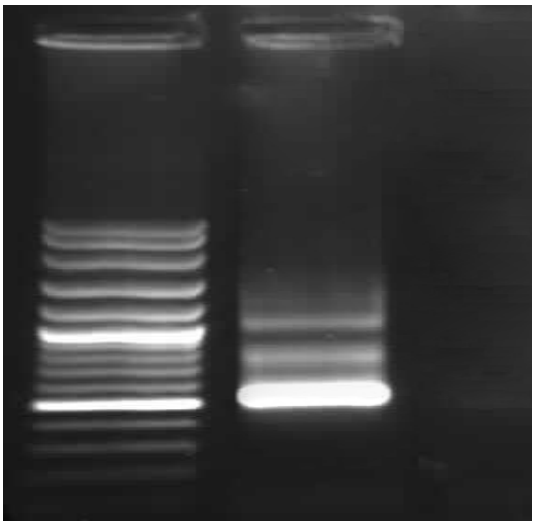

Fig. 2. Gel electrophoresis of PCR products for *fim* Uropathogenic virulence factors. M: 100bp ladder and 1: positive samples

strains of *E. coli*, P =0.036 in the incidence of *afa* gene between the imipenem resistant and imipenem susceptible strains and P =0.047 in the incidence of *hly* gene between the imipenem resistant and imipenem susceptible strains. There were significant differences in the incidence of *cnf1* (P =0.039), *pap* (P =0.041) and *fim* (P =0.049) genes between imipenem resistant and imipenem susceptible strains of *E. coli*.

DISCUSSION

The present study specified that 65.62% of urine samples of hospitalized patients were infected with *E. coli*. In keeping with this, 7.61% of these *E. coli* strains were resistant to imipenem and it is so substantial, while high efficacy of imipenem for treatment of UTIs has been reported previously from Iran (Farshad *et al*., 2012; Japoni *et al*., 2008). Higher levels of resistance in *E. coli* strains against imipenem has been reported by Poirel *et al*. (2004) and Martinez-Martinez *et al*. (2000). Therefore, judicious use of antibiotics is required by clinicians.

The present study showed significant assortment in the distribution of virulence factors among imipenem resistant and imipenem susceptible strains of UPEC. Imipenem resistant strains harbored higher amounts of *sfa*, *afa* and *hly* factors, while those that were imipenem susceptible harbored higher amounts of *fim*, *cnf1* and *pap* factors. Contrariwise, the prevalence of the *sfa, afa* and *hly* genes was lower in the imipenem susceptible strains. This finding suggests that imipenem resistance could be directly associated with decreased prevalence of *pap*, *cnf1* and *fim* virulent genes, as suggested in a previous studies (Vila *et al*., 2002; Farshad *et al*., 2010). Horcajada *et al*. (2005) reported that nalidixic acid resistance strains of *E. coli* was associated with a significantly decreased prevalence of *sfa*, *hly* and *cnf1* virulence factors. It has also been shown by Johnson *et al*. (1988) that the presence of the *pap* determinants was also significantly associated with a lack of antimicrobial agent resistance, as it was the case with the *hly* determinants.

One possible explanation for the high prevalence of specific virulence factors in imipenem resistant and/or imipenem susceptible strains is that *E. coli* virulence factors are often located on plasmids or transposons and these virulence factors can be linked to antibiotic resistance genes (Boerlin *et al*., 2005; Travis *et al*., 2006), creating the potential for antimicrobial use targeted at UTIs to coselect for virulence genes and worsen disease severity. In despite of this, there were some previously published data showed that susceptible strains of *E. coli* harbored higher amount of virulence factors (Horcajada *et al*., 2005; Rosengren *et al*., 2009; Vila *et al*., 2002). The extent that imipenem resistant strains of *E. coli* from people with UTIs carries linked virulence genes has not been studied extensively.

Vila *et al*., (2002) reported that quinolone-resistant UPEC strains harbored lower virulence factors than susceptible strains. They showed that *hly* and *cnf1* factors were less prevalent (P <0.05) in nalidixic acid–resistant than in nalidixic acid–susceptible *E. coli* strains from patients with either pyelonephritis (14.3% vs. 52.4%) or cystitis (0% vs. 31.0%). Vila *et al*., (2002) indicated that among *E. coli* strains causing cystitis, *fim* expression was less prevalent (P <0.05) in the nalidixic acid–resistant group (55.2%) than in the nalidixic acid–susceptible group (86.2%). Lower prevalence of *iucD* gene was observed among nalidixic acid–susceptible strains than nalidixic acid–resistant strains of UPEC (Vila *et al*., 2002). It has been shown that quinolone resistant UPEC strains express fewer virulence factors than quinolone susceptible strains (Farshad *et al*., 2010).

According to the studies on the rapport between uropathogenic virulence genes which have copies on chromosome and plasmid, and imipenem sensitivity or resistance, it seems that genetic mechanisms related to chromosomes which are involved in producing resistance to imipenem can induce the expurgation of pathogenicity islands (PAIs) genes from chromosome (Bagel *et al*., 1999). On the other hand, genetic relationship studies like virulence factors profiling left any possibility out for gaining of imipenem resistance by *E. coli* strains that naturally lack these virulence factors and then spread in a clonal fashion (Ott *et al*., 1993). Although all premises virtually solidly have suggested that becoming resistant to antibiotic may affect the absence or presence of these factors, another description for this marvel might be that some genetic intermediaries involved in the expression of virulence genes may conquer the expression of the promoter compulsory for

resistance to imipenem. Although further studies are academically warranted to merge the findings.

In conclusion, taking into account the findings, we propose that *cnf1*, *pap* and *fim* genes are predominant in imipenem susceptible strains of UPEC. However, more complimentary studies in larger groups of UPEC strains are necessary to confirm these finding.

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