Antibiotic resistance profile and virulence genes of uropathogenic *Escherichia coli* isolates in relation to phylogeny

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Abstract. *Escherichia coli* (*E. coli*) strains are the major cause of urinary tract infections (UTI) and belong to the large group of extra-intestinal pathogenic *E. coli*. The purposes of this study were to determine the antibiotic resistance profile, virulence genes and phylogenetic background of *E. coli* isolates from UTI cases. A total of 137 *E. coli* isolates were obtained from UTI samples. The antimicrobial susceptibility of confirmed isolates was determined by disk diffusion method against eight antibiotics. The isolates were examined to determine the presence and prevalence of selected virulence genes including *iucD*, *sfa/focDE*, *papEF* and *hly*. ECOR phylo-groups of isolates were determined by detection of *yjaA* and *chuA* genes and fragment TspE4.C2. The antibiogram results showed that 71% of the isolates were resistant to cefazolin, 60.42% to co-trimoxazole, 54.16% to nalidixic acid, 36.45% to gentamicin, 29.18% to ciprofloxacin, 14.58% to cefepime, 6.25% to nitrofurantoin and 0.00% to imipenem. Twenty-two antibiotic resistance patterns were observed among the isolates. Virulence genotyping of isolates revealed that 58.39% isolates had at least one of the four virulence genes. The *iucD* gene was the most prevalent gene (43.06%). The other genes including *sfa/focDE*, *papEF* and *hly* genes were detected in 35.76%, 18.97% and 2.18% isolates, respectively. Nine combination patterns of the virulence genes were detected in isolates. Phylotyping of 137 isolates revealed that the isolates fell into A (45.99%), B1 (13.14%), B2 (19.71%) and D (21.16%) groups. Phylotyping of multidrug resistant isolates indicated that these isolates are mostly in A (60.34%) and D (20.38%) groups. In conclusion, the isolates that possessed the *iucD*, *sfa/focDE*, *papEF* and *hly* virulence genes mostly belonged to A and B2 groups, whereas antibiotic resistant isolates were in groups A and D. *Escherichia coli* strains carrying virulence factors and antibiotic resistance are distributed in specific phylogenetic background.

INTRODUCTION

Extra-intestinal pathogenic *Escherichia coli* (ExPEC) is one of the main agents of morbidity and mortality in human through the world (Kudinha *et al.*, 2012). These agents are responsible for cholecystitis, traveler's diarrhoea and other clinical infections such as neonatal meningitis, pneumonia, pyelonephritis, cystitis, intestinal and urinary tract infection (UTI) which is the most significant disease in ExPEC group (Anvarinejad *et al.*, 2012; Madappa *et al.*, 2012).

There are wide diversity of researches on the virulence factors and genotypic characteristics of *E. coli* isolates. Uropathogenic *Escherichia coli* (UPEC) show a variety of virulence factors (VFs). These VFs participate in colonization, invasion and, consequently, reduction of the host immunity responses (Anvarinejad *et al.*, 2012; Agarwal *et al.*, 2013). The strains with virulence associated genes consist

of adhesions, toxins, iron acquisition systems, biofilms and other virulence factors (Johnson *et al.*, 2012; Agarwal *et al.*, 2013). The adhesions such as S and P fimbriae play the major roles in the pathogenicity of *E. coli* strains by colonization stage and overcoming host immunity (Bahalo *et al.*, 2013). Adhesion to uroepithelial cells by P fimbriae is an important step for the beginning and expansion of UTI (Matiuzzi da Costa *et al.*, 2008; Bahalo *et al.*, 2013). S fimbriae showed attachment efficiency to epithelial and endothelial cells of the lower human urinary tract (Bien *et al.*, 2012). Studies have shown *papEF* and *sfa/focDE* are essential for cystitis and/or pyelonephritis (Bahalo *et al.*, 2013). The iron chelator molecules gain iron from host cells by aerobactin (*iucD*) which is an important factor for growth of extra-intestinal pathogens of *E. coli* (Bien *et al.*, 2012). Haemolysin enzyme is secreted by uropathogen *E. coli* species. Haemolysin (HlyA) causes tissue damages, facilitates bacterial distribution and participates in bacterial pathogenesis (Davis *et al.*, 2005; Johnson *et al.*, 2007).

Escherichia coli strains were assigned into four major phylogenetic groups: A, B1, B2 and D and six subgroups A_0 and A_1 (for phylogenetic group A), D_1 and D_2 (for phylogenetic group D), and $B2_2$ and $B2_3$ (for phylogenetic group B2) (Deschamps *et al.*, 2009; Abdul-Razzaq *et al.*, 2011). Extra-intestinal pathogenic strains usually fell into B2 and a lesser extent group D (Abdul-Razzaq *et al.*, 2011; Platell *et al.*, 2012). The phylogenetic group B2 isolates mostly carries more virulence genes than other isolates (Kawamura–Sato *et al.*, 2010; Abdul-Razzaq *et al.*, 2011).

Increased and non-controlled anti-microbial administrations can lead to acquired resistance in extra-intestinal infections of *E. coli* which can lead to increased healthcare costs through the world, morbidity and mortality. Therefore, in order to control the prevalence of resistant antimicrobial agents, selection of an antibiotic according to symptoms and directed by urinalysis is suitable for uncomplicated cystitis but should be altered based on culture results for more severe infections (Sheerin, 2011). The relationship between phylogenetic background and antibiotic resistance indicates that group B2 is the more susceptible than A, B1 and D phylogenetic groups (Kawamura-Sato *et al.*, 2010). The aim of this study was to determine the antibiotic resistance profile, virulence genes and phylogenetic background of *E. coli* isolates from patients with UTI in Kerman, Iran by PCR.

MATERIAL AND METHODS

Bacterial strains
One hundred and thirty seven urinary samples were obtained from admitted patients to four different hospitals during October to December, 2009 in Kerman province, Iran (south-eastern). The samples were from 37 males and 100 females (100). Their age ranged from <15 years old (45), 15 to 30 years old (38), 30 to 45 years old (26) and 45 to 70 years old (28). The samples were cultured on MacConkey agar and EMB (Biolife Laboratories, Milano, Italy). In order to confirm *E. coli* isolates, standard biochemical and bacteriological tests were used. From each sample one confirmed *E. coli* isolate was selected and stored in Luria-Bertani broth (In vitrogen, Paisley, Scotland) with 30% sterile glycerol at -70ºC.

PCR and reference strains
Strains were DNA extracted from *E. coli* isolates and reference strains by lysis method. For this study, the presence of the *papEF*, *sfa/focDE*, *hly* and *iucD* genes were determined by PCR method as described previously (Yamamoto, 2007) and phylo-genetic groups (A, B1, B2, and D) of *E. coli* isolates were carried out by triplex PCR by Clermont *et al.* (2000). *Escherichia coli* reference strains ECOR62 (*chuA*+, *yjaA*+ and Tspe4.C2+), 28C (*hly*+), J96 (*sfa/focDE*+, *papEF*+) and A30 (*iucD*+) were used as positive controls. *Escherichia coli* strain MG1655 was used as a negative control. All the reference strains were from Microbiology Department of Ecole Nationale Vétérinaire Toulouse. The primers used for detecting sequences encoding virulence genes and

phylogenetic groups are described in Table 1.

Antimicrobial susceptibility testing
The antimicrobial susceptibility of *E. coli* isolates were determined by disk diffusion method following the Clinical Laboratory Standards Institute (CLSI) guidelines using commercial antimicrobial disks (Mast. Co., UK). The antibiotic disks used in this study were cefazolin (30 µg), ciprofloxacin (5 µg), co-trimoxazole (1.25/23.75 µg), nitrofurantoin (300 µg), gentamicin (10 µg), imipenem (10 µg), cefepime (30 µg) and nalidixic acid (30 µg). *Escherichia coli* ATCC 25922 was used as a quality control strain. In literal terms, multidrug resistant (MDR) means resistant to more than one antimicrobial agent *in vitro* (Magiorakos *et al.*, 2012).

RESULTS

PCR assays revealed that 137 isolates belonged to A 45.99% (63 isolates), B1 13.14% (18), B2 19.71% (27) and D 21.16% (29) phylogenetic groups. Phylotyping of isolates indicated that the isolates could be categorized into six subgroups: A_0 (33.59%), A_1 (12.41%), $B2_2$ (1.45%), $B2_3$ (18.25%), D_1 (6.56%), and D_2 (14.60%). Subgroups A_0 and $B2_3$ were the most prevalent among the examined isolates.

From the disk diffusion assay, the current study found that 71% of the *E. coli* UTI isolates were resistant to cefazolin. Different percentages of antibiotic resistance were recorded against co-trimoxazole (60.42%), nalidixic acid (54.16%), gentamicin (36.45%), ciprofloxacin (29.18%), cefepime (14.58%), and nitrofurantoin (6.25%). However, none of the isolates were resistant to imipenem. One isolate was sensitive to ciprofloxacin, cefepime, gentamicin, nitrofurantoin, and co-trimoxazole, while intermediate to cefazolin and nalidixic acid. Twenty two antibiotic resistance patterns were detected in relation to phylogenetic groups (Table 2). Phylotyping of antibiotic resistant isolates demonstrated that these isolates mostly belonged to A (41.28%) and D (19.03%) groups (Table 2). Phylogenetic background of quinolone-resistant (resistant to ciprofloxacin and nalidixic acid patterns) and MDR isolates including all of the antibiotic resistant patterns except cefazolin, nalidixic acid and co-trimoxazole patterns indicated that these isolates were mostly in groups A (60.34%) and D (20.38%).

Among antibiotic resistant patterns, cefazolin+nalidixic-acid+co-trimoxazole and cefazolin+co-trimoxazole were the most prevalent patterns, which had at least one of the *iucD*, *sfa/focDE*, *papEF* and *hly* genes (Table 3).

Virulence genotyping of isolates indicated that 80 (58.39%) isolates had at least one of virulence-associated genes. The aerobactin encoding genetic marker (*iucD*) was the most prevalent gene (43.06%) in the isolates, while 36 (26.28%) isolates were positive for this gene in combination with other genes, and 23 (16.78%) isolates had just the *iucD* gene (Table 4). Phylotyping of 59 isolates possessed the genetic marker *iucD* showed that the isolates distributed in four phylogenetic groups including A (31 isolates), B1 (6), B2 (12) and D (10) phylogenetic groups.

The P and S fimbriae coding genes were detected in 26 (18.97%) and 49 (35.76%) isolates of UTI isolates, respectively. PCR assays revealed that 31.38% isolates possessed *sfa/focDE* gene in combination with other genes and 6 (4.37%) isolates just exhibited the *sfa/focDE* gene. Among the isolates positive for *papEF* gene, 6 (4.37%) of the isolates possessed *papEF* gene exclusively, whereas 14.59% of isolates showed the genetic marker *papEF* in the present of other genes (Table 4). Twenty six isolates were positive for *papEF* gene, which segregated in A (6 isolates), B1 (2), B2 (14) and D (4) phylogenetic groups. Also 49 isolates were positive for *sfa/focDE* gene that fell into A (19 isolates), B1 (3), B2 (19) and D (8) phylogenetic groups. The positive isolates for virulence genes were distributed in six phylogenetic subgroups (Table 5).

The genetic marker hemolysin (*hly*) was present in 2.18% isolates in combination with other genes (Table 4) that fell into A (1 isolate) and B2 (2) phylogenetic groups.

Table 1. Oligonucleotide primers used in this study

Primer	Primer sequence (5'-3')	Product size (bp)
sfa/focDE	CTCCGCAGAACTGGGTGCATCTTA CGCAGGAGTAATTACAAACCTGGCA	410
papEF	GCAACAGCAACGCTGGTTGCATCA AGAGAGAGCCACTCTTATACGGACA	336
iucD	TACCGGATTGTCATATGCAGACCGT AATATCTTCCTCCAGTCCGGAGAAG	602
hly	AACAAGGATAAGCACTGTTCTGGCT ACCATATAAGCGGTCATTCCCGTCA	1177
yjaA	TGAAGTGTCAGGAGACGCTG ATGGAGAATGCGTTCCTCAAC	211
TspE4.C2	CTGGCGAAAGACTGTATCAT CGCGCCAACAAAGTATTACG	152
chuA	GACGAACCAACGGTCAGGAT TGCCGCCAGTACCAAAGACA	279

Table 2. Antibiotic resistance patterns in relation to phylogenetic background

Antibiotic resistance patterns	Phylogenetic group				Total
	A	B1	B2	D	
CZ	7	6	5	3	21
TS	2	–	–	–	2
NI	1	–	–	–	1
CZ, TS	7	–	4	4	15
CZ,GM	1	–	7	5	13
CZ, NA	4	–	–	3	7
NA,TS	1	–	–	2	3
CZ, NA, TS	8	4	6	2	20
CZ,GM, TS	–	2	2	–	4
CIP, CZ, NA	1	–	–	–	1
CIP, CZ, NA,TS	3	1	2	–	6
CZ,GM, NA,TS	2	–	–	2	4
CIP, CZ, GM, TS	1	–	–	–	1
CIP, CZ, GM, NA	1	2	–	–	3
CPM, CZ, GM, NI	2	–	–	–	2
CPM, CZ, GM, TS	3	–	–	–	3
CIP, CZ, NA, NI, TS	2	–	–	2	4
CIP, CZ, GM, NA, TS	4	–	2	2	8
CPM, CZ, GM, NA, TS	–	2	2	–	4
CIP, CPM, CZ, GM, NA	–	–	–	4	4
CIP, CPM, CZ,GM, NA,TS	3	1	1	1	6
CIP, CPM, CZ, GM, NA, NI, TS	4	–	–	–	4
Total	57	18	31	30	136

CIP ciprofloxacin, CPM cefepime, CZ cefazolin, GM gentamicin, NA nalidixic acid, NI nitrofurantoin, TS co-trimoxazole

Table 3. Antibiotic resistance patterns in relation to virulence genes

Antibiotic resistance patterns	Virulence genes				Total
	iucD	*sfa/focDE*	*papEF*	*hly*	
CZ	8	3	6	1	18
TS	–	–	–	–	–
NI	–	–	–	–	–
CZ, TS	8	8	4	1	21
CZ,GM	1	8	8	–	17
CZ, NA	1	1	1	–	3
NA,TS	–	1	1	–	2
CZ, NA, TS	10	8	4	–	22
CZ,GM, TS	–	1	–	–	1
CIP, CZ, NA	1	–	–	–	1
CIP, CZ, NA,TS	9	6	–	–	15
CZ,GM, NA,TS	3	3	–	–	6
CIP, CZ, GM, TS	–	–	–	–	-
CIP, CZ, GM, NA	1	–	–	–	1
CPM, CZ, GM, NI	1	1	–	–	2
CPM, CZ, GM, TS	–	–	–	–	–
CIP, CZ, NA, NI, TS	–	–	–	–	–
CIP, CZ, GM, NA, TS	3	–	–	–	3
CPM, CZ, GM, NA, TS	1	–	–	–	1
CIP, CPM, CZ, GM, NA	–	–	–	–	–
CIP, CPM, CZ,GM, NA,TS	9	6	–	–	15
CIP, CPM, CZ, GM, NA, NI, TS	–	–	–	–	–
Total	56	46	24	2	128

CIP ciprofloxacin, CPM cefepime, CZ cefazolin, GM gentamicin, NA nalidixic acid, NI nitrofurantoin, TS co-trimoxazole

Table 4. Virulence genes and their combination patterns detected in 137 *E. coli* isolates from UTI

Combination patterns	Gene				Total No (%)
	sfa/focDE	*papEF*	*iucD*	*hly*	
sfa/focDE	+	–	–	–	6 (4.37%)
papEF	–	+	–	–	6 (4.37%)
iucD	–	–	+	–	23 (16.78%)
iucD/sfa	+	–	+	–	24 (17.51%)
papEF/sfa	+	+	–	–	10 (7.29%)
papEF/iucD	–	+	+	–	1 (0.72%)
iucD/hly	–	–	+	+	1 (0.72%)
papEF/iucD/sfa	+	+	+	–	8 (5.83%)
papEF/iucD/sfa/hly	+	+	+	+	1 (0.72%)
Negative	–	–	–	–	57 (41.60%)
Total No (%)	49 (35.76%)	26 (18.97%)	59 (43.06%)	3 (2.18%)	137 (100%)

Table 5. Virulence genes in UTI isolates in relation to group/subgroups phylogenetic

Gene	A_0	A_1	B1	$B2_2$	$B2_3$	D_1	D_2	Total N (%)
PapEF	3 (11.54)	3 (11.54)	2 (7.70)	–	14 (53.84)	–	4 (15.38)	26 (100.00)
iucD	21 (35.60)	10 (16.95)	6 (10.17)	–	12 (20.34)	1 (1.69)	9 (15.25)	59 (100.00)
sfa/focDE	16 (32.66)	3 (6.12)	3 (6.12)	–	19 (38.77)	–	8 (16.33)	49 (100.00)
hly	–	1 (33.33)	–	2 (66.67)	–	–	–	3 (100.00)
Total N (%)	40 (29.20)	17 (12.41)	11 (8.03)	2 (1.46)	45 (32.85)	1 (0.72)	21 (15.33)	137 (100.00)

According to the results, nine patterns of gene combination were detected. Among 137 *E. coli* isolates, *iucD+sfa/focDE* (17.51%) and *papEF+sfa/focDE* (7.29%) patterns were the most prevalent in the isolates, respectively (Table 4).

DISCUSSION

This study addressed the important roles of virulence genes in *E. coli* UTI. The virulence associated genes play important roles in development of extra-intestinal *E. coli* infections (Wang *et al.*, 2002). These isolates can be defined into four major phylogenetic groups: A, B1, B2, and D. In *E. coli* extra-intestinal infections, B2 group was more predominant than three other phylogenetic groups (Rijavec *et al.*, 2008). The relationship between phylogenetic groups and resistance has shown that group B2 strains were more susceptible to antibiotics than were groups A, B1 and D strains. On the other hand, many studies reported that quinolone-resistant and fluoroquinolone-resistant strains fell into specific phylogenetic background, such as A and D (Moreno *et al.*, 2006; Kawamura-Sato *et al.*, 2010).

In the current study, siderophore encoding gene (*iucD*) was found to be the most prevalent isolate (43.8%), compared to 25.9% reported by Tiba *et al.* (2008) and 46% reported by Marrs *et al.* (2002), while other studies reported 91% and 59% (Piatti *et al.*, 2008; Rijavec *et al.*, 2008). Aerobactin siderophore systems would be necessary to obtain iron from iron chelator molecules (Ejrnæs, 2011).

In the present study, fimbrial genes were examined in the isolates. These genes involve in potential binding to epithelial cells in lower human urinary tract. The results showed that more isolates harboured the *sfa/focDE* (36.5%) gene compared to the *papEF* (17.5%) gene. In the other parts of the world, some researches have determined the significance of these virulence genes in pathogenicity. Tiba *et al.* (2008) showed that 27.8% and 32.7% of isolates possessed *sfa/focDE* and *papEF* genes, respectively. In Brazil, prevalence of *sfa/focDE* gene in combination with *papEF* gene was 83.3% (Santo *et al.*, 2006). In a study on Iran, 27.15 and 14.6% of *E. coli* isolates possessed *pap* and *sfa* genes (Farshad *et al.*, 2009).

This study demonstrated that hemolysin encoding sequence (*hly*) was present in a small percentage (2.2%) of isolates. It is believed that *hly* is more common amongst invasive uropathogenic strains than healthy fecal isolates (Katouli & Vollmerhausen, 2010). In India, 4.7% of *E. coli* isolates associated with acute cystitis in women were positive for hemolysin genetic marker (Agarwal *et al.*, 2013), whereas Yamamato (2007) showed most frequencies of isolates (41.2%) possessed *hly* gene.

PCR analyses of phylotyping determination showed that *E. coli* isolates mostly fell into group A, followed by B2 and D. The finding of the present study is in agreement with study by Piatti *et al.* (2008), which reported A and B1 phylogenetic groups were the most prevalent in the isolates, respectively. In contrast, several previous investigations have indicated that most of the isolates belonged to B2 followed by D and B1 groups (Kawamura-Sato *et al.*, 2010; Choi *et al.*, 2012).

ExPEC strains, which cause UTI, commonly belong to the groups B2 or D, whereas strains of the B2 and D groups involved more virulence-associated genes

than strains of the A and B1 groups (Nowrouzian *et al.*, 2006). Grude *et al.* (2007) surveyed *E. coli* isolates from Norwegian and Russian females with significant bacteruria who presented with clinical signs of UTI; Russian isolates fell into more often to A phylogenetic group and possessed fewer virulence genes than did Norwegian isolates, whereas groups B2 and D were significant among the Norwegian isolates and were higher in number of virulence genes than did Russian isolates (Grude *et al.*, 2007).

In the present study, most of the isolates were resistant to cefazolin, co-trimoxazole and nalidixic acid. Farshad *et al.* (2009) showed that highest antibiotic resistances were towards imipenem, nitrofurantuin and ciprofloxacin, while nalidixic acid and gentamycin were more sensitive. In accordance, in a study in Brazil, co-trimoxazole, nalidixic acid, gentamycin, ciprofloxacin and cephalothin were the most resistant in the isolates (Oliveria *et al.*, 2011).

Several previous investigations have demonstrated that quinolone-resistant and MDR are associated with a shift towards non-B2 phylogenetic groups (Johnson *et al.*, 2005; Rijavec *et al.*, 2008). Based on the results nitrofurantoin, ciprofloxacin, co-trimoxazole and nalidixic acid belonged to A and D groups whereas *E. coli* isolates that were sensitive to imipenem fell into B2 phylogenetic group. Moreover, Moreno *et al.* (2006) found that isolates susceptible to quinolone, fluoroquinolone and trimethoprim/ sulfamethoxazole were significantly associated with phylogenetic group B2, whereas resistant isolates exhibited shifts to A and D groups (Moreno *et al.* 2006).

In conclusion, the *E. coli* strains were segregated into different phylogenetic groups, and the B2 phylogenetic group represented the majority of strains involved in different extra-intestinal infections. The current findings indicated that the virulence associated genes were mostly distributed in A and B2 groups, while antibiotic resistant strains were mainly in A and D phylogenetic groups. B2 phylogenetic group strains were less prone to be resistant to antibiotics than non-B2 strains. Therefore, although *E. coli*

strains carried virulence factors and acquired the resistance by mutation, which were distributed in some specific phylogenetic background, the relationship was complex.

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