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Virulence genotyping of *Escherichia coli* isolates from diarrheic and urinary tract infections in relation to phylogeny in southeast of Iran

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Abstract. The purpose of this study was to determine the prevalence of virulence genes and phylogenetic groups/subgroups of *Escherichia coli* (*E. coli*) isolates from diarrheic and urinary tract infections (UTI) cases in Rigan area, southeast of Iran. One hundred thirty five *E. coli* were isolated from diarrheic (90 isolates) and urinary tract infections (45 isolates) samples. The confirmed isolates were examined to detect the phylogenetic group/subgroups and a selection of virulence genes including *iucD*, *sfa/focDE*, *afaIBC*, *papEF*, *hly*, *cnfI* and *cdtI* by PCR. The examined isolates belonged to four phylogenetic groups A (42.2%), B1 (14.1%), B2 (10.4%), and D (33.3%). Among 135 tested bacteria, 62.22% of diarrheic and 30.37% of UTI isolates had at least one of the virulence genes. In the diarrheic isolates *iucD* (47.77%) was the most prevalent gene. The other genes including *sfa/focDE*, *afaIBC*, *papEF* and *cnfI*/*cdtI* genes were detected in 15, 13, 11 and one diarrheic isolates respectively. None of the diarrheic isolates were positive for *hly* gene. Out of 45 UTI isolates 28.88% were positive for *iucD*, 13.33% for *cnfI*, 11.11% for *afaIBC*, 11.11% for *papEF*, 6.66% for *sfa/focDE* and 4.44% for *cdtI* genes. Several combination patterns of the virulence genes were detected in diarrheic and UTI isolates. In conclusion, the prevalence of virulence genes in diarrheic and UTI isolates differ according to phylogenetic groups, although B2 and D phylotypes have an accumulation of virulence associated genes.

INTRODUCTION

Pathogenic *Escherichia coli* (*E. coli*) strains are known to cause intestinal and extra-intestinal infections in human. A wide variety of infectious diseases could be caused by *E. coli* strains, including urinary tract infection, septicemia, newborn meningitis, central nervous system and respiratory system infections (Zhang *et al.*, 2002; Arisoy *et al.*, 2008; Abdul-Razzaq *et al.*, 2011). Several bacterial agents can cause diarrheal and urinary tract infections, among these *E. coli* strains are detected as an important cause of morbidity and mortality of diarrhoea and UTI throughout the world (Arisoy *et al.*, 2008; Bisi-Johnson *et al.*, 2011).

The hypothesis express, that uro-pathogenic *E. coli* (UPEC) strains than non-pathogenic strains acquiring new virulence factors by pathogenicity islands (Mladin *et al.*, 2009). The virulence potential of extra-intestinal pathogenic *E. coli* (ExPEC) is associated with many virulence factors (VFs) including P, S fimbriae and afimbrial adhesin encoding genes *papEF*, *sfa/focDE*, *afaIBC*, haemolysin (*hly*), aerobactin (*iucD*), cytotoxic necrotizing factor (*cnf*I), cytolethal distending toxin *(cdtI*). These VFs play important roles in the

pathogenicity of *E. coli* strains by colonizing the key anatomical sites, affect the host physiology and invade host tissues (Costa *et al.*, 2008; Farshad *et al.*, 2010; Abdallah *et al.*, 2011). Pathogenic *E. coli* adherence is a prerequisite step for initial and successful colonization of specific host uroepithelial cells (Melican *et al.*, 2011; Oliveira *et al.*, 2011). The toxin types including alfa-haemolysin, cytotoxic necrotizing factor 1 (CNF1) and cytolethal distending toxin (CDT) are associated with extra-intestinal infections. CNF1 and HLY are involved in host cell damage in urinary tract. Haemolysin stimulates sloughing of the uroepithelial cells and bladder hemorrhage while CNF1 causes bladder inflammation and submucosal oedema in mice (Costa *et al.*, 2008; Oliveira *et al.*, 2011). Five different *cdt* alleles have been identifed in *E. coli* strains that encode CDT-I, CDT-II, CDT-III, and CDT-IV (Ghanbarpour & Oswald, 2009).

The aerobactin system which is encoded by a five gene operon (*iucA*, *iucB*, *iucC*, and *iucD*, *iutA*) is an expression of the iron acquisition systems. It has been found that the mentioned system is able to utilize siderophores for scavenging iron from the environment for the ability to colonize and persist in host iron-poor niches such as the urinary tract (Ghanbarpour & Oswald, 2009; Oliveira *et al.*, 2011).

Phylogenetic studies show that *E. coli* strains belong to four major phylogenetic groups, designated A, B1, B2, and D and six subgroups (A_0, A_1, $B2_2$, $B2_3$, D_1 and D_2) (Carlos *et al.*, 2010; Choi *et al.*, 2012). The commensal *E. coli* strains tend to associate within phylogenetic groups A and B1 and pathogenic *E. coli* strains carry more virulence genes into phylogenetic groups B2 and D. Extra-intestinal infections are mainly due to *E. coli* strains belonging to group B2 and to a lesser extent, group D (Mokracka *et al.*, 2011; Bashir *et al.*, 2012).

The objectives of this study were to detect the virulence encoding genes of *E. coli* isolates from diarrheal and urinary tract infections and to determine the ECOR phylogenetic groups/subgroups of isolates in Rigan area, southeast of Iran by PCR.

MATERIALS AND METHODS

Sampling and bacteriological examinations

This study was under taken on 135 *E. coli* isolates (90 diarrhoea and 45 urine samples) Rigan area, southeast of Iran. The diarrheic and urine samples were obtained from April 2010 to August 2011 for the isolation and characterization of *E. coli*. The bacteriological samples were obtained from patients of Rigan area referred to Pasteur hospital. Patients provide informed consent by agreeing to participate and signing an Informed Consent Form. The *E. coli* isolates were from diarrheic and urine samples of patients aged between 5 to 33 years old. The *E. coli* isolates were recovered from both female (71) and male (64). The isolates were identified as *E. coli* based on standard bacteriological and biochemical tests. From each sample one confirmed *E. coli* isolate was selected and was stored in Luria-Bertani broth (Invitrogen, Paisley, Scotland) with 30% sterile glycerol at -80°C.

PCR and reference strains

Escherichia coli reference strains ECOR62 (*chuA*, *yjaA* and Tspe4.C2), 28C (*hly*, *cnf1*), J96 (*sfa/focDE*, *papEF*), A30 (*afaIBC*, *iuc*D) and E6468/62 (*cdt-I*) 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. DNA was extracted from *E. coli* isolates and reference strains by lysis method to NaOH.

Specific PCR primers for *papE-F*, *afaIBC*, *sfa/focDE*, *hly* and *iucD* were previously described by Yamamoto *et al.* (Yamamoto *et al.*, 1995) and those for *cnf1* and *cdt-I* genes by Toth *et al.* (Toth *et al.*, 2003) and phylogenetic groups (A, B1, B2, and D) were described by Clermont *et al.* (Clermont *et al.*, 2000). Sequences and sizes of PCR products are shown in Table 1.

Table 1. Oligonucleotide primers used in this study

Gene	Primer Sequence (52-32)	Product size (bp)
afaIBC	GCT GGG CAG CAA ACT GAT AAC TCT CCAT CAA GCT GTT TGT TCG TCC GCC G	750 bp
sfa/focDE	CTC CGG AGA ACT GGG TGC ATC TTA CCGG AGG AGT AAT TAC AAA CCT GGC A	410 bp
papEF	GCA ACA GCA ACG CTG GTT GCA TCA TAGA GAG AGC CAC TCT TAT ACG GAC A	336 bp
hly	AAC AAG GAT AAG CAC TGT TCT GGC TACC ATA TAA GCG GTC ATT CCC GTC A	1177 bp
iucD	TAC CGG ATT GTC ATA TGC AGA CCG TAAT ATC TTC CTC CAG TCC GGA GAA G	602 bp
cnfI	GGG GGA AGT ACA GAA GAA TTATTG CCG TCC ACT CTC ACC AGT	1112 bp
cdtI	CAA TAG TCG CCC ACA GGAATA ATC AAG AAC ACC ACC AC	412 bp
yjaA	TGA AGT GTC AGG AGA CGC TG ATG GAG AAT GCG TTC CTC AAC	211 bp
TspE4C2	GAG TAA TGT CGG GGC ATT CA CGC GCC AAC AAA GTA TTA CG	152 bp
chuA	GAC GAA CCA ACG GTC AGG AT TGC CGC CAG TAC CAA AGA CA	279 bp

RESULTS

Phylogenetic analysis revealed that 135 *E. coli* isolates segregated in phylogenetic group A (42.2%), B1 (14.1%), B2 (10.4%), and D (33.3%). PCR assays for phylotyping of 90 diarrheic isolates indicated that the strains distributed in four phylogenetic groups including 35 isolates (38.88%) in A, 18 isolates (20%) in B1, 14 isolates (15.56%) in B2, and 23 isolates (25.56%) in D group. PCR assays of 45 UTI *E. coli* isolates showed that 4 isolates (8.89%) belonged to A, 7 isolate (15.56%) to B1, 19 isolates (42.22%) to B2 and 15 isolates (33.33%) to D phylogenetic groups. Phylotyping of diarrheic isolates showed that the isolates fell into six subgroups A_0, A_1, $B2_2$, $B2_3$, D_1 and D_2, whereas most of them belonged to A_1 25.55% (23 isolates) phylogenetic subgroup. Forty five *E. coli* isolates of UTI fell into four phylogenetic subgroups A_0, A_1, $B2_2$, $B2_3$, D_1 and D_2, whereas the $B2_3$ 26.66% (12 isolates) was the most prevalent subgroup (Table 2).

Genotyping of 135 *E. coli* isolates revealed that 62.22% (84 isolates) of diarrhoea and 30.37% (41 isolates) of UTI had at least one of the examined virulence-associated genes. The aerobactin coding gene (*iucD*) was the most prevalent gene in both of diarrhoea and UTI isolates (41.48%). This gene was detected in 47.77% and 28.88% of diarrheic and UTI isolates respectively. Forty three *iucD* positive isolates from diarrheic cases fell into A (17 isolates), B1 (8), B2 (5) and D (13) and 13 UTI isolates belonged to B1 (3), B2 (4) and D (6) phylogenetic groups (Table 3).

Haemolysin coding gene was the second most prevalent (15.55%) virulence gene found in the UTI isolates and was present among the isolates from B1 (1 isolates), B2 (4) and

Table 2. Distribution of diarrhoea and UTI isolates in phylogenetic groups/subgroups

Phylo		Diarrhoea isolates		UTI isolates	
group	subgroup	N	(%)	N	(%)
A		35	(38.88)	4	(8.89)
	A_0	12	(13.33)	3	(6.66)
	A_1	23	(25.55)	1	(2.22)
B1		18	(20)	7	(15.56)
B2		14	(15.56)	19	(42.22)
	$B2_2$	13	(14.44)	7	(15.55)
	$B2_3$	1	(1.11)	12	(26.66)
D		23	(25.56)	15	(33.33)
	D_1	15	(16.66)	6	(13.33)
	D_2	8	(8.88)	9	(20)

Table 3. Virulence genes in diarrhoea and UTI isolates in relation to group/subgroups phylogenetic

Gene	Diarrhoea isolates								UTI isolates							
	A_0	A_1	B1	$B2_2$	$B2_3$	D_1	D_2	total	A_0	A_1	B1	$B2_2$	$B2_3$	D_1	D_2	total
iucD	6	11	8	4	1	9	4	43	–	–	3	1	3	2	4	13
papEF	1	4	1	1	–	1	3	11	–	1	–	–	2	1	1	5
sfa/focDE	2	4	5	2	–	1	1	15	–	–	1	–	2	–	–	3
afaIBC	–	3	2	4	–	4	–	13	1	–	–	–	2	–	2	5
cnfI	–	1	–	–	–	–	–	1	1	–	–	2	1	2	–	6
cdtI	–	–	1	–	–	–	–	1	–	–	–	1	–	1	–	2
hly	–	–	–	–	–	–	–	–	–	–	1	2	2	–	2	7
Negative	3	–	1	2	–	–	–	–	1	–	1	1	–	–	–	–
Total	9	23	17	11	1	15	8	–	2	1	6	6	12	6	9	–

D (2) phylogenetic groups. Of all the diarrheic isolates investigated, none were positive for the *hly* gene (Table 3).

The genetic markers *papEF*, *sfa/focDE* and *afaIBC* were more prevalent in diarrheic than in UTI isolates. The P, S and Afa fimbriae coding genes were detected in 12.22%, 16.66% and 14.44% of diarrheic isolates respectively. Prevalence of *papEF*, *sfa/focDE* and *afaIBC* genes in UTI isolates were 11.11%, 6.66% and 11.11% respectively. Among 135 *E. coli* 16 (11.85%) isolates were positive for *papEF* gene. Eleven *papEF* positive diarrheic isolates belonged to A (5 isolates), B1 (one), B2 (one) and D (4) groups, whereas the five *papEF* positive UTI isolates fell into A (one isolate), B2 (2) and D (2) phylogenetic groups (Table 3).

Prevalence of *sfa/focDE* gene in both groups of isolates was 13.33%. Among 90 diarrheic isolates 15 bacteria possessed *sfa/focDE* gene, which were segregated in A (6 isolates), B1 (5), B2 (2) and D (2) groups, while 3 *sfa/focDE* positive UTI isolates fell into B1 (one) and B2 (2) phylogenetic group (Table 3).

PCR assays revealed that 18 (13.33%) isolates possessed *afaIBC* gene. Thirteen *afaIBC* positive *E. coli* isolates from diarrheic samples fell into 4 phylogenetic groups A, B1, B2 and D, whereas five isolates of UTI fell into A (one isolate), B2 (2 isolates) and D (2) phylogenetic groups (Table 3).

Of the 135 examined isolates, *cnf1* and *cdt-I* genes were detected in 7 (5.18%) and 3 (2.22%) isolates respectively. The CNF1 and

Table 4. Combination of virulence genes in diarrhoea and UTI isolates in relation to phylogenetic groups

Combination of virulence gene	human with diarrhoea isolates					UTI isolate				
	A	B1	B2	D	total	A	B1	B2	D	total
iucD, papEF, sfa/focDE	1	–	–	–	1	–	–	–	1	1
iucD, papEF	1	–	–	2	3	–	–	1	1	2
iucD, sfa/focDE	2	–	1	–	3	–	–	1	–	1
papEF, sfa/focDE	–	–	–	1	1	–	–	–	1	1
iucD, hly	–	–	–	–	–	–	–	1	–	1
papEF, hly	–	–	–	–	–	–	–	1	–	1
cnfI, cdtI	–	–	–	–	–	–	–	1	–	1
Total	4	–	1	3	–	–	–	5	3	–

CDT-I encoding genes were detected in one isolate (1.11%) in the diarrheic samples that belonged to A and B1 phylogenetic groups. Among urine samples 6 (13.33%) isolates were positive for *cnf1* that belonged to A (one isolate), B2 (3) and D (2) phylogroups. The *cdt-I* gene was positive for 2 isolates (4.44%) of urine samples which, fell into B2 and D phylogenetic groups (Table 3).

Several combination patterns of the virulence genes were detected in diarrheic and UTI isolates (Table 4).

DISCUSSION

Pathogenic *E. coli* strains have the potential to cause a wide variety of infectious diseases. Studying the genetic diversity and genetic relationships between pathogenic *E. coli* isolates such as diarrheic and UTI isolates is important for deciphering the molecular basis for the pathogenesis. A wide variety of VFs could be playing a potential role in extra-intestinal and intestinal infections *E. coli* isolates. These genes were chosen for this study, because of their ability to cause disease. On the other hand the known virulence genes were most often associated with specific phylogenetic groups specially B2 and D groups (Carlos *et al*., 2010).

According to the results the aerobactin coding gene *(iucD)* was the most prevalent gene in both of diarrheic and UTI isolates. The apparent increment in iron-acquisition genes among the *E. coli* isolates may be an indicator of the importance of iron in the pathogenesis. In the current study *iucD* gene was detected in 47.77% of diarrheic and 28.88% of UTI isolates. In Japan and Slovenia, the prevalence of *iucD* gene were detected in 59.3% and 91% of *E. coli* isolates from urine samples, respectively (Rijavec *et al*., 2008; Kawamura-Sato *et al*., 2010). In a study conducted in Michigan, 49.46% of *E. coli* isolates from UTI and 30.77% of commensal rectal isolates possessed aerobactin encoding sequences (Zhang *et al*., 2002).

The *hly* gene was detected in UTI isolates (15.55%), while all of the diarrheic isolates were negative for haemolysin. In Japan, 20.5% of UTI isolates were positive for *hly* gene (Kawamura-Sato *et al*., 2010). It is believed that, the high prevalence of *hly* gene in UTI isolates could not be interpreted as a sure sign that this gene is not associated with pathogenesis. Marrs *et al*. (2002) indicated that *hly* gene is more frequently found in UTI than diarrheic isolates.

In this study several fimbrial genes were examined. According to the results, *sfa/focDE* gene was detected in the 16.66% and 6.66% of diarrheic and UTI isolates respectively. In USA, 7% of diarrheic isolates were positive for S fimbriae (Johnson *et al*., 2007). In a study, *sfa/focDE* gene was reported in 13.92% of asymptomatic bacteriuria isolates, whereas all of the UTI isolates were negative for this gene (Wang *et al*., 2009). Perez *et al*. (2010) showed a high percentage of extra-intestinal-associated strains harboring intestinal-associated virulence genes. This

genetic heterogeneity in strains suggested a high potential for horizontal gene transfer. In the present study five of the UTI isolates were positive for the *papEF* and *afaIBC* genes, whereas 12.22% and 14.44% of diarrheic isolates were positive for P and Afa fimbriae encoding genes. Marrs *et al.* (2002) reported that several fimbrial types such as P-pili family (*pff*), $papG_{J96}$, $papG_{AD}$ and S fimbriae occurred at lower frequencies in diarrheic than UTI isolates. The finding of the present study is in agreement with a study that detected 4% *pap*, *afaI* and *cnf1* and 1% *sfa* genetic markers of UTI isolates in Turkey (Arisoy *et al.*, 2008).

Two other genes were found to occur in UTI isolates but did not occur in the majority of diarrheic tested isolates. A small percentage of diarrheic isolates were positive for the *cnf1* and *cdt1* genes (1.11%), whereas 13.33% and 4.44% of UTI isolates were positive for CNF1 and CDT-I encoding genes, respectively. Sannes *et al.* (2004) showed most frequencies of isolates from extra-intestinal *E. coli* than fecal isolates were positive for *cnf1* and *cdtB* gene sequences. Bouzari *et al.* (2005) reported that 37.5% and 25.3% of diarrheic isolates were positive for four types of *cdt* (*cdt-I*, *cdt-II*, *cdt-III* and *cdt-IV*) and two types of *cnf* (*cnf1* and *cnf2*).

PCR results of phylogenetic determination, showed that diarrheic isolates mostly fell into group A, followed by D. However, according to the results UTI isolates mostly belonged to B2 and D phylogenetic groups. Similar to this result, previous reports indicated that the most ExPEC strains belonged to group B2, although some fell into group D (Johnson *et al.*, 2005; Moulin-Schouleur *et al.*, 2007). Perez *et al.* (2010) indicated that diarrhea-associated *E. coli* isolates are dispensing among all phylogenetic groups. Ejrnaes *et al.* (2011) reported that phylogenetic group B2 was the predominant phylo-group among the primary infection *E. coli* from women having significan bacteriuria, followed by D, A and B1. The majority of studies reported a similar distribution of phylogenetic group among UPEC; however one study indicated a predominance of phylogenetic group A among UPEC isolates in Russia (Johnson *et al.*, 2005; Moulin-Schouleur *et al.*, 2007; Grude *et al.*, 2007). Nowrouzian *et al.* (2006) reported that 38% of resident *E. coli* strains from the colonic microbiota belonged to phylogenetic group B2. Conversely, phylogenetic group A was more common among transient strains.

Pathogenicity of extra-intestinal *E. coli* has been associated with presence of virulence genes in relation to some phylogenetic groups. Group B2 strains have an accumulation of VF genes (Kawamura-Sato *et al.*, 2010; Choi *et al.*, 2012). In the present study, the distribution of virulence genes in each phylo-group indicated that diarrheic isolates fell into A and D groups whereas majority of UTI isolates segregated in the phylo-groups B2 and D. A study on fecal and urine *E. coli* isolates established the presence of ten virulence genes that occurred more frequently in either group B2 and D (Zhang *et al.*, 2002).

This study provided relevant information on the prevalence of virulence associated genes and phylogenetic background of *E. coli* isolates from diarrhoea and UTI in Rigan, Iran. Totally the results of the present study confirm that *E. coli* strains of the phylogenetic group A and D have superior prevalence.

In conclusion and regarding to the prevalence and distribution of examined virulence genes in phylo-groups, the pathogenic potential of groups B2 and D strains may cause extra-intestinal and intestinal infections. Further studies are needed to examine the isolates in B2 and D phylo-groups in more detail in comparison to their virulence associated genes to further refine the definition of pathogenic *E. coli*.

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