Detection of Enterohemorrhagic *Escherichia coli* Related Genes in *E. coli* Strains Belonging to B2 Phylogroup Isolated from Urinary Tract Infections in Combination with Antimicrobial Resistance Phenotypes

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ABSTRACT

Background: This study was conducted to detect the prevalence of EHEC virulence genes and antimicrobial resistance profile of *Escherichia coli* strains belonging to B2 phylogroup implicated in Urinary tract infections in Semnan, Iran.

***Methods*:** From 240 urine samples 160 *E. coli* strains were isolated, biochemically. Then, *E. coli* isolates were examined by Multiplex-PCR for phylogenetic typing and detection of virulence genes (*hly*, *stx1*, *stx2*, *eae*) associated with Enterohemorrhagic *E. coli*. Finally, Antimicrobial resistance of *E. coli* isolates were characterized using Disk Diffusion method.

***Results*:** From 160 *E. coli* isolates, 75 strains (47%) were assigned to B2 phylogenetic group and prevalence of virulence genes were as follow: *hly* (21.3%), *stx1* (16%), *stx2* (10.6%) and *eae* (6.7%), subsequently. Phenotypic antimicrobial resistance of B2 isolates showed that all isolates were sensitive to Meropenem and Furazolidone and then highest frequency of resistance was observed to Streptomycin, Oxytetracycline, Neomycin, Nalidixic acid and Ampicillin (98.7% to 49.3%). Also low resistance prevalence was observed in case of Ceftizoxime, Lincospectin, Imipenem, Chloramphenicol and flurefenicole (16% to 1.3%).

***Conclusion*:** The data suggest a high prevalence of antibiotic resistance in UPEC strains belonging to B2 phylogroup even for the antimicrobials using in pet and farm animals and their potential to cause EHEC specific clinical symptoms which may represent a serious health risk since these strains can be transmitted to GI tract and act as a reservoir for other uropathogenic *E. coli* and commensal strains.

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Introduction

Escherichia coli (*E. coli*) strains belonging to enterobacteriaceae family, are normal habitant of digestive tract in a wide range of hosts including humans. Various strains of this species have been divided into different pathotypes based on the pathogenesis and virulence factors properties (1). Pathogenic strains of *E. coli* cause various diseases in humans, including several types of diarrhoea, urinary tract infections, sepsis, and meningitis (2). Urinary tract infections (UTIs) are the most common extra-intestinal *E. coli* infections and are caused by Uropathogenic *E. coli* (UPEC) (2, 3). Diarrhea associated with the hemolytic-uremic syndrome (HUS) and neurologic complications is generally caused by *E. coli* strains related to enterohemorrhagic (EHEC) pathotype, potentially able to produce Shiga toxins and such strains usually harbour the enterocyte effacement pathogenicity island, which facilitates colonization of the bacterium into the gastrointestinal tract (4). *E. coli* strains based on some genetic markers (*chuA*, *yjaA*, and *TspE4.C2*) are divided into four main phylogenetic groups A, B1, B2 and D, and various studies have demonstrated that extra intestinal pathogenic *E. coli* (ExPEC) strains such as UPEC and EHEC mainly belong to groups B2 and D (5). Various studies have demonstrated that intestinal pathogenic *E. coli* strains are the most common agents causing UTI's and transmission of these bacteria happen via sexual intercourse, the anatomical short distance from the urethra to the anus in women and poor observation of preventive criteria (6, 7). The aim of the present study was to evaluate the distribution of EHEC virulence factors related genes (Intimin; Shigatoxin1, 2 and Hemolysin) in the UPEC strains related to B2 phylogroup isolated from urinary tract infections to assess their genetic relationships and evaluation of their potential to cause HUS and dysentery, because strains associated to B2 phylogroup are the major cause of UTI's; hemorrhagic colitis and HUS (8-10).

The aim of the present study was to detect the EHEC related virulence genes in UPEC isolates to assess their importance in transmission of these genes to other E. coli pathotypes and antimicrobial resistance patterns in B2 phylogroup as the major uropathogenic strains.

Materials and Methods

Strains collection

E. coli isolation and identification was carried out from out-patients (25-45 years old/both sexes) suspected to suffering urinary tract infections based on urologist's diagnosis referring to diagnostic laboratories in Semnan, Iran, according to the protocol described by Bonadio et al. (11). Briefly, urine samples were streaked on Mac Conkey Agar and incubated 24 hours in 37 ºC and after incubation period, the *E. coli* strains were identified by standard biochemical tests. A specimen was considered positive for UTI in the light of the number of yielded colonies ($\geq 10^5$ cfu/mL) and the cytology of the urine through microscopic detection of bacteriuria and PMNs (≥8 leukocytes/mm3). Then, stock cultures were prepared from the *E. coli* isolates and stored in Luria-Bertani broth with 15% (v/v) glycerol at -20 °C until genotyping.

DNA extraction, Phylogenetic & Virulence genotyping

Genomic DNA was extracted from *E. coli* strains based on alkaline lysis of bacteria (12) and phylogenetic group of isolates were characterized using a modified Triplex PCR-based assay optimized for detection of *chuA*, *yjaA*, and *tspE4. C2* gene markers as previously described by Derakhshandeh et al. (13). Then, the detection of

Enterohemorrhagic *E. coli* virulence genes (*eaeA*, *hlyA*, *stx*1 and *stx*2) was carried out using a Tetraplex-PCR method as described by Paton & Paton in 1998 (14). The primers sequences used in this study are present in Table 1.

Antimicrobial susceptibility test

All *E. coli* strains were tested for antimicrobial susceptibility using the agar disk diffusion method to 8 antibiotic classes (Twelve antibiotics) purchased from PADTAN TEB Co, (Iran) including Penicillin (Ampicillin); Cefalosporin (Ceftizoxime); Tetracycline (Oxytetracycline); Aminoglycosides (Streptomycin, Lincospectin, Neomycin); Carbapenem (Imipenem, Meropenem); Quinolone (Nalidixic acid); Nitrufuran (Furazolidone) and Thiamphenicole (Flurefenicole, Chloramphenicole) according to the guidelines provided by the Clinical and Laboratory Standards Institute (CLSI) (15, 16). Finally distribution of antimicrobial resistance versus various virulence genotypes were analyzed using Chi-Square (X2) and Fisher's Exact Test at 95% of confidence and P = 0.05 of significant level.

Results

In the present study 160 *E. coli* strains were isolated from urinary tract infections, biochemically. Then, distribution of the phylogenetic groups within these strains showed that 75 strains (47%) belong to B2 phylogenetic group as the predominant phylogroup followed by groups D (30%), A (15.5%) and B1 (7.5%), subsequently.

The detection of EHEC related virulence factors using Tetraplex-PCR in B2 strains revealed that 31 (41.3%) of all B2 isolates (n = 75) were positive for at least one of the virulence genes tested. In the tested strains, *hly* (21.3%) was the most common virulence gene identified, followed by *stx1* (16%), *stx2* (10.6%) and *eae* (6.7%), respectively. Also virulence genotyping revealed that following gene combinations (*stx1*+; *stx2*+; *stx1*+/*stx2*+), (*eae* + *stx2*), (*stx2* + *eae* + *hly*) and (*hly* + *eae*) were observed in 24%, 6.7%, 1.3% and 2.7% of B2 strains, respectively (Table 2).

A high percentage of B2 phylogroup strains examined in this study exhibited resistance to Streptomycin, Oxytetracycline, Neomycin, Nalidixic acid and Ampicillin (98.7%, 85.3%, 78.7%, 65.3% and 49.3%, respectively). Ceftizoxime resistance was observed in 16% of B2 isolates. Furthermore, a lower percentage of resistance was identified to Lincospectin (8%), Imipenem (6.7%), Chloramphenicol (6.7%) and flurefenicole (1.3%). However, all the isolates were sensitive to Meropenem and Furazolidone. Among the 18 *stx* positive (*stx1*+; *stx2*+; *stx1*+/*stx2*+) strains, all were resistant to Streptomycin and sensitive to Furazolidone, Imipenem and Meropenem. Also high resistance was observed in Oxytetracycline, Neomycin, Nalidixic acid and Ampicillin (15; 11; 9; 9 isolates, respectively) and high sensitivity to Chloramphenicole, Flurefenicole, Lincospectin and Ceftizoxime (17; 17; 17; 16) was observed in *stx* positive B2 strains. Within *eae*+ strain, antimicrobial resistance was observed about Streptomycin, Ampicillin, Oxytetracycline, Neomycin and Nalidixic acid (5; 4; 4; 3; 1) and they were sensitive to all other antibiotics tested. Antimicrobial resistance profile of *hly*+ strains in B2 phylogroup was as follow: all resistant to Streptomycin and sensitive to Furazolidone, Meropenem and Flurefenicole. Also in *hly*+ isolates resistance was observed in case of Neomycin, Oxytetracycline, Nalidixic acid, Ampicillin, Chloramphenicole, Lincospectin, Imipenem and Ceftizoxime (14; 13; 10; 8; 1; 1; 1; 1, respectively) (Table-2). The only significant difference in antimicrobial resistance between different virulence genotypes was observed in case of Nalidixic acid between *eae*+*stx2* genotype and B2 strains without any detected genes demonstrating that resistance to this antibiotic is lower in *eae*+*stx2* group versus strains harbouring none of the virulence genes, significantly (P = 0.0366).

Discussion

Bacterial infection of urogenital tract is one of the most frequent infectious diseases and various documents reveal that *E. coli* is the predominant etiology of UTI, worldwide because 70-95% of UTI's are caused by UPEC (17-19). In this study, a total of 160 *E. coli* strains were isolated from 240 urine samples (67%) of patients suffering urinary tract infections based on urologist's diagnosis and referred to medical diagnostic laboratories in Semnan, Iran and these results confirm *E. coli* as the common agent of UTI's in the region. *E. coli* isolates were tested to assess the phylogenetic group and presence of 4 EHEC-virulence genes and susceptibility to 12 antibiotics.

Most *E. coli* strains causing UTI are distributed within phylogroup B2, and to a lesser expansion group D, while mainly other groups include commensal and low pathogenic strains (20). In our study, 47% of *E. coli* isolates from UTI cases were assigned to B2 phylogroup which it is in parallel with other investigations results. It has been stated UPEC strains have virulence factors like P & S fimbriae, afimbrial adhesion apparatuses and Iron acquisition systems through pathogenicity islands for the invasion and colonization of urinary tract and typically belong to these two prevalent phylogroups (8, 21). Distribution of mentioned virulence factors within our *E. coli* isolates from UTI cases demonstrated that these factors are more prevalent in B2 phylogroups in comparison to other strains, significantly (8).

Enterohemorrhagic *E. coli* serve as a critical pathotype of Shiga toxin-producing *E. coli* (STEC) group and can cause severe disease in host organs ranging from haemorrhagic colitis and diarrhea in enteric tract to thrombocytopenic purpura and hemolytic uremic syndrome (22). Diverse serotypes of EHEC are linked with mentioned clinical disorders in human and the ability to produce Shiga toxins and induction attaching and effacing lesions are considered essential in the pathogenesis of EHEC strains (9, 23, 24). Different studies have demonstrated that STEC strains mainly are belonging to phylogroup B2 (25). In the present study *stx*1 and *stx*2 were observed in 16% and 10.6% of tested *E. coli* isolates belonging to B2 group. Some studies demonstrate that strains producing only *stx2* are more pathogenic potentially than strains harbouring *stx*1 or even strains producing both *stx1* and *stx2*, while these two subgroups share approximately 55% amino acid homology (26, 27). It is of note that most HUS-associated clinically relevant STEC isolates produce *stx*2, but rarely, *stx*1 is highly significant in some regions like Europe and in vitro (LD50 in mice) studies show that *Stx*2 can be approximately 400 times more toxic than *Stx*1(27, 28). About 10% of our B2 strains were positive for *stx*2, showing the potential of such strains to cause EHEC related clinical signs but the fact that EHEC strains for induction of HUS and hemorrhagic colitis symptoms need to adhere to enteric mucosa via Intimin (eae) and presence of *eae* only in 6.7% of B2 strains shows that not all strains producing shigatoxins are able to colonize and induce EHEC disorders in the host because several enteric pathogens can produce shigatoxins and EHEC strains in addition to shigatoxins need to have other virulence gene clusters like LEE (Locus of Enterocyte Effacement) and type III secretion pathways (T3SS) (23, 29), so detection of LEE & T3SS related virulence genes reveals their capability to these severe disorders more obviously. Also 21% of our B2 strains were positive for *hly* gene as a virulence factor responsible for HUS and HC symptoms. Various documents show that *hly* and hyl operons are interfering in hemolysin production, activation and secretion from bacterial cell. The hyl operon is found on a plasmid of EHEC O157:H7, while the *hly* operon is often located adjacent to the P fimbrial genes on the same pathogenicity island on the chromosome of UPEC strains. Hyl proteins are responsible for hemolysin export and activation (30-32). For assessment of the relation of our B2 strains having *hly* gene to EHEC pathotype, detection of hyl ORF's (Open Reading Frame's) seems to be necessary.

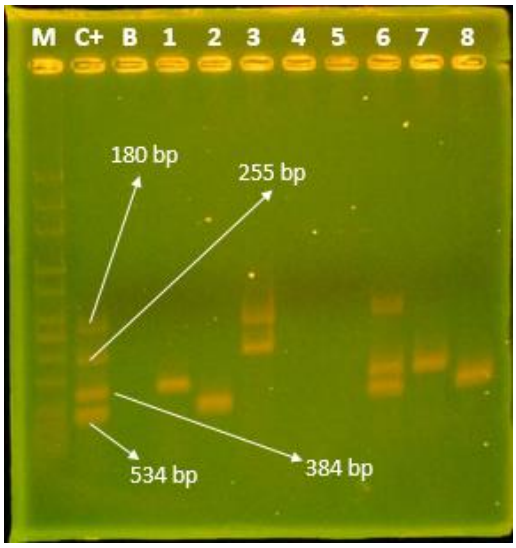

Figure 1. Gel electrophoresis results of defined virulence genes (eae, *stx*1, *stx*2 and *hly*A) in B2 strains from UTI cases. M: Marker; C+: Positive Control; B: Blank (Negative Control); 1-8: Samples showing different virulence genotypes.

Table 1. Polymerase chain reaction primers used to detect phylogenetic groups and selected genes of Enterohemorrhagic *E. coli* in strains belonging to B2 phylogroup.

	Primer sequence (5’ to 3’)	Product size (bp)	References
chuA	GACGAACCAACGGTCAGGAT	279	(5)
	TGCCGCCAGTACCAAAGACA		
yjaA	TGCCGCCAGTACCAAAGACA	211	(5)
	ATGGAGAATGCGTTCCTCAAC		
TspE4.C2	GAGTAAGTTCGGGGCATTCA	152	(5)
	CGCGCCAACAAAGTATTACG		
Stx1	ATAAATCGCCATTCGTTGACTAC	180	(14)
	AGAACGCCCACTGAGATCATC		
Stx2	GGCACTGTCTGAAACTGCTCC	255	(14)
	TCGCCAGTTATCTGACATTCTG		
eaeA	GACCCGGCACAAGCATAAGC	384	(14)
	CCACCTGCAGCAACAAGAGG		
hlyA	GCATCATCAAGCGTACGTTCC	534	(14)
	AATGAGCCAAGCTGGTTAAGCT		

There has been a serious increase in resistance of uropathogens as well *E. coli* to antibiotics over the last years and territorial variations of resistance to antibiotics may be explained in part by different regional antimicrobial administrations (33). Antimicrobial resistance phenotypes of B2 strains obtained in the present study showed sensitivity or low resistance to Meropenem, Imipenem and Ceftizoxime while high resistance rate was observed in case of Streptomycin, Neomycin, Nalidixic acid and Ampicillin as antimicrobial choices for treatment of human infections. Today, some antimicrobials are not used any more in human practice such as Furazolidone and Chloramphenicol but some are still in use in animal husbandry like Oxytetracycline, Lincospectin and Flurefenicole. There are several documents showing the livestock's as the major reservoir of *E. coli* for human and usually transmission of such strains happen via consumption of foods with animal origin and *E. coli* strains originating from animals can colonize in the human enteric organs getting a member of human flora (34-36). *E. coli* strains circulating in livestock are exposed to a great selective pressure because in different geographical parts a wide range of antibiotics are used in food animals, so antimicrobial resistance is increasing and resistance genes can spread on mobile genetic elements such as transposons, integrons and plasmids (37). The results obtaining from antimicrobial resistance phenotypes in the present study shows that there may be *E. coli* strains with animal origin in human populations of our study region causing urinary tract infections and to confirm this fact fully genotyping of the isolates from human and animal cases is recommended. To prevent more antibiotic resistance in human strains, as a role, antibiotics such as Streptomycin, Nalidixic acid and Ampicillin, showing high resistance should not be selected as first choice therapeutics by Urologist's. Although among the all antibiotics tested, Meropenem and Imipenem were effective medicines against all B2 strains (probably due to their infrequent use), but the administration of any recommended antimicrobial should be based on the prior execution of susceptibility testing results of an isolated *E. coli* strain.

Our results show that *E. coli* strains acquiring EHEC related genes may be distributed within B2 phylogroup as the major Uropathogenic *E. coli* and probably with potential to induce HUS and HC in GI tract and transmit to environment and other hosts via oral-faecal routes. Also antibiotic resistance profile of B2 strains in combination with EHEC virulence genes are in parallel with the fact that livestock especially ruminants are an important reservoir of such strains for human.

Conclusion

Considering the virulence and antimicrobial resistance gene transfer mechanisms between pathogenic and nonpathogenic *E. coli* strains, this research may offer useful insights for both human and veterinary clinicians, with the additional scope of increasing farmer's attention to their critical role in the control of the transmission of pathogenic strains of *E. coli*.

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Conflict of interest

None declared conflicts of interest.

Financial disclosure

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