Phylogenetic grouping and pathotypic comparison of urine and fecal *Escherichia coli* isolates from children with urinary tract infection

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Abstract

The aim of this study was to investigate the phylogenetic background and to assess *hlyD* (involved in the secretion of haemolysin A) and *intI₁* (encoding a class 1 integrase) in *Escherichia coli* isolates derived from urinary and fecal specimens. A total of 200 *E. coli* isolates was collected from patients presenting with urinary tract infection (UTI) during September 2009 to September 2010 and screened for *hlyD* and *intI₁* genes by polymerase chain reaction (PCR). Phylogenetic analysis showed that *E. coli* is composed of four main phylogenetic groups (A, B1, B2 and D) and that uropathogenic *E. coli* (UPEC) isolates mainly belong to groups B2 (54%) and D (34%) whereas group A (44%) and D (26%) are predominant among commensal *E. coli* isolates. In this study, *hlyD* was present in 26% of UPEC and 2% of commensal *E. coli* isolates. However, hemolytic activity was detected for 42% of UPEC and 6% of commensal *E. coli* isolates (*p < 0.05*). *intI₁* gene was more frequently expressed in UPEC (24%) in comparison with commensal *E. coli* isolates (12%). Resistance to aztreonam, co-trimoxazole and cefpodoxime were frequently found among UPEC isolates whereas commensal *E. coli* isolates were commonly resistant to co-trimoxazole, nalidixic acid and cefotaxime. Concluding, a considerable difference between UPEC and commensal *E. coli* isolates was observed regarding their phylogenetic groups, presence of class 1 integron and *hlyD* gene, hemolysin activity and resistance pattern. The detection of class 1 integrons and *hlyD* gene was higher among UPEC compared with commensal *E. coli* isolates. These findings may contribute for a better understanding of the factors involved in the pathogenesis of UPEC.

Key words: *Escherichia coli*, urinary tract infection (UTI), phylogenetic typing groups, *hlyD*, *intI₁*.

Introduction

Urinary tract infections (UTIs) currently rank among the most prevalent infectious diseases worldwide, with chronic and recurrent infections being especially problematic (Blango and Mulvey, 2010; Sabate *et al.*, 2006). The primary etiologic agents associated with UTIs are strains of uropathogenic *Escherichia coli* (UPEC) (Sivick and Mobley, 2010). Nonetheless, UPEC isolates express a wide spectrum of virulence and fitness factors that aid in successful colonization of the mammalian urinary tract (Manges *et al.*, 2004). Although often categorized as extracellular pathogens, UPEC can in fact invade a number of host cell types, including the terminally differentiated superficial facet cells and less mature intermediate and basal epithelial cells that comprise the stratified layers of the bladder urothelium. Host cell invasion is proposed to facilitate both the establishment and persistence of UPEC within the urinary tract (Johnson *et al.*, 2005; Mulvey *et al.*, 2000).

Extra-intestinal pathogenic and commensal *E. coli* typically differ in phylogenetic group and virulence attrib-
utes. Previous studies have shown that pathogenic extraintestinal E. coli isolates primary belong to phylogenetic group B2 and, to a lesser extent, group D, whereas commensal E. coli isolates belong to groups A and B1. Moreover, pathogenic extraintestinal isolates harbour specialized virulence factors, i.e., traits that confer pathogenic potential, which are infrequent among commensal isolates (Johnson et al., 2001; Sabate et al., 2006).

Currently, about 50 different cassettes associated with resistance genes, can be found in different classes of integrons. An integron is a two component gene capture and dissemination system, first discovered due to their rapid dissemination of antibiotic resistance, which can be found in plasmids, chromosomes and transposons. The first component consists of a gene encoding a site specific recombinase along with a specific site for recombination, while the second component comprises fragments of DNA called gene cassettes which can be incorporated or shuffled. A cassette may encode genes for antibiotic resistance, although most genes in integrons are uncharacterized. Integrons act as receptors of antibiotic resistance cassettes (Kovalevskaya, 2002).

Hemolysin is a cytolytic protein toxin secreted by most hemolytic E. coli isolates. In addition of lysing erythrocytes, hemolysin is a toxin for a wide range of host cells which may result in inflammation, tissue injury, and impaired host defenses. It should be mentioned that monocytes and granulocytes are highly susceptible to hemolysin cytotoxicity, whereas lymphocytes are relatively resistant. Exposure of polymorphonuclear leukocytes (PMNLs) to hemolysin stimulates degranulation and releases of leukotrienes accompanied by ATP; causes marked morphologic alterations; and impaired chemotaxis and phagocytosis (Johnson, 1991). Hemolysin production correlates closely with the toxicity of clinical E. coli isolates for PMNLs. Hemolysin stimulates superoxide anion and hydrogen peroxide release and oxygen consumption by renal tubular cells as well as histamine release from mast cells and basophils (Johnson, 1991).

The aim of this study was to determine the phylogenetic type of uropathogenic and commensal E. coli, isolated from patients with UTI in Mofid Childrens’ Hospital, Tehran, Iran. In addition, the prevalence of hemolytic activity, and the assessment of hlyD gene (involved in hemolysin production) and of class I integron (a genetic element associated with antibiotic resistance) were also investigated, in order to provide additional information about E. coli virulence profiles.

Material and Methods

Specimens and patients

A total of 200 E. coli isolates were analyzed from 100 children patients of both sexes (85% female, 15% male) aged between 2-12 years with UTI (70% pyelonephritis, 30% cystitis). Of these, 100 were derived from midstream clean catch urine and 100 were from stool specimens of the patients presenting with community acquired UTI who have attended the nephrology ward of Mofid Childrens’ Hospital, Tehran, Iran, during September 2009 to September 2010. The project was approved by the local Ethics Committee for Human Researches.

Samples were derived from fresh midstream urine, cultured (0.01 mL) on MacConkey agar (Sisco Research Laboratories Pvt. Ltd., USA) as well as Sheep blood agar and incubated at 37 °C for 24 h. Urine bacteria included in this study were from cultures yielding > 10^5 CFU/mL. Cultures with < 10^5 CFU/mL were further investigated only if relevant history of fever, chills, flank pain, pyuria, antibiotic intake, structural abnormalities, diabetes mellitus or any other immunocompromised state was present.

Specimens from stool samples were cultured on Trypticase soy agar (Kanto Chemical Co., Inc., Japan) with 5% sheep blood and MacConkey agar. The predominant isolate on each plate (one colony) and all morphologically distinct colonies were identified and stored for further analysis, as described by Plos (1995) and Foxman (2002). Two-three colonies, cultured on sheep blood as well as on MacConkey agar, from each stool and urine sample, were selected for molecular examination (Moreno et al., 2006).

Antimicrobial susceptibility test

Susceptibility to nitrofurantoin (300 μg), ciprofloxacin (5 μg), nalidixic acid (30 μg), amoxicillin (10 μg), augmentin (30 μg), gentamicin (120μg), ceftazidime (30 μg), cefpodoxime (10 μg), aztreonam (30 μg), imipenem (10 μg), amikacin (30 μg), co-trimoxazole (25 μg) and cefotaxime (30 μg) were determined by disc diffusion assays (BBL Sensi-Disc, USA) modified by the Kirby-Bauer method using CLSI criteria (nonfastidious groupings M2-disk diffusion M100). For the purpose of analysis, intermediate susceptibility was considered as susceptible (Schlagler et al., 2002).

DNA extraction

DNA was extracted using the protocol described previously (Sabarinath et al., 2011). The isolates were cultured on MacConkey agar plates for 24 h. One to two colonies were resuspended in 0.5 mL sterile distilled water. The cells were lysed by heating at 95 °C for 10 min and the supernatant was harvested by centrifugation at 12,000 rpm (8000 g) for 5 min. The supernatant was used as the source of the template DNA.

PCR amplification

Briefly, this consists of a 300 nM concentration of each oligonucleotide primer (BIO NEER, Takapouzist.co, AccuOligo®), web: http://www.bioneer.com) 5.5 mM MgCl2; 200 mM (each) deoxynucleoside triphosphates
dATP, dCTP, dGTP, and dUTP; and 0.125 U of Taq DNA polymerase (GENET BIO, Prime Taq™ DNA polymerase, type:G-1002, URL:www.genetbio.com).

**Phylogenetic typing group**

Phylogenetic grouping of the *E. coli* isolates was determined by a simple, rapid PCR-based technique (Clermont et al., 2000) that uses a combination of three DNA markers (*chuA*, *yjaA* and DNA fragment tspE4.C2), generating 279, 211 and 152-bp fragments, respectively. A triplex PCR was performed using the six primers in a single reaction. The results of these three amplifications allowed the classification of *E. coli* isolates into one of the major phylogenetic groups: A, B1, B2 or D. *E. coli* strain RS218, which belongs to phylogenetic group B2, was used as a control (Dhakal et al., 2008).

**Hemolytic activity and hlyD gene detection**

*E. coli* isolates were inoculated on 5% sheep blood agar plates and incubated overnight at 37 °C. The plates were then examined for the presence of a partial or total hemolytic activity (alpha or beta) (Forbes et al., 2007).

PCR was performed using the *hlyD* gene (904 bp) primers: F CTCCGGTACGTGAAAAGGAC: (Tm = 55.4 °C), R GCCCTGATTACTGAAGCCTG: (Tm = 55.7 °C) in a single reaction (Rodriguez-Siek et al., 2005).

**Class 1 integron detection**

Isolates were analyzed by polymerase chain reaction (PCR) amplification techniques to determine whether a class 1 integron was present. Integrons were detected by PCR amplification of a class 1 integrase-specific fragment of the *intI1* gene. The primer sequences used were *intI1*-F: GGTCAAGGATCTGGATTTGG, R: ACATGCGTGTAAATCATCGTC) in a single reaction (Rodriguez-Siek et al., 2005).

**Statistical analysis**

Statistical analysis was performed by using the Fisher exact and chi-square tests. The threshold for statistical significance was a p value of < 0.05.

**Results**

**Pattern of antimicrobial resistance among *Escherichia coli* isolates**

High-level resistance to azteronam (78%), co-trimoxazole (61%), cefpodoxime (48%) were found among UPEC while commensal *E. coli* isolates showed increased resistance to co-trimoxazole (82%), nalidixic acid (27%) and cefotaxime (27%). Resistance pattern of UPEC and commensal *E. coli* isolates were presented in Tables 1 and 2.

**Table 1 - Antibiotic resistance pattern among phylogenetic groups in Uropathogenic *E.coli* isolates from children with community acquired UTI.**

| Antibiotic   | A   | B1  | B2   | D   | Total |
|--------------|-----|-----|------|-----|-------|
| Augmentin    | -   | -   | 12%  | 2%  | 14%   |
| Cefotaxime   | 3%  | 2%  | 39%  | 1%  | 45%   |
| Co-trimoxazole| -  | 1%  | 50%  | 10% | 61%   |
| Imipenem     | -   | -   | -    | -   | 0%    |
| Nitrofurantoin| -  | -   | 2%   | -   | 2%    |
| Amoxicillin  | -   | -   | 16%  | -   | 16%   |
| Gentamicin   | 2%  | -   | 19%  | 1%  | 22%   |
| Ciprofloxacin| -   | -   | 7%   | 1%  | 8%    |
| Amikacin     | 1%  | 1%  | 5%   | 1%  | 8%    |
| Ceftazidime  | -   | -   | 12%  | -   | 12%   |
| Cefpodoxime  | 2%  | 6%  | 38%  | 2%  | 48%   |
| Nalidixic acid| -  | -   | 9%   | -   | 9%    |
| Azteronam    | 1%  | -   | 48%  | 29% | 78%   |

**Table 2 - Antibiotic resistance pattern among phylogenetic groups in commensal *E.coli* isolates from children with community acquired UTI.**

| Antibiotic   | A   | B1  | B2   | D   | Total |
|--------------|-----|-----|------|-----|-------|
| Augmentin    | -   | -   | 7%   | 2%  | 9%    |
| Cefotaxime   | 2%  | -   | 16%  | 9%  | 27%   |
| Co-trimoxazole| 29%| 14%| 16% | 23%| 82% |
| Imipenem     | -   | -   | -    | -   | 0%    |
| Nitrofurantoin| -  | -   | 6%   | 2%  | 8%    |
| Amoxicillin  | 9%  | 1%  | 8%   | 2%  | 20%   |
| Gentamicin   | -   | -   | 1%   | 11% | 12%   |
| Ciprofloxacin| 1%  | -   | 1%   | 3%  | 5%    |
| Amikacin     | -   | -   | 3%   | -   | 3%    |
| Ceftazidime  | -   | -   | 3%   | 4%  | 7%    |
| Cefpodoxime  | 2%  | 1%  | 15%  | 1%  | 19%   |
| Nalidixic acid| -  | 2%  | 16%  | 9%  | 27%   |
| Azteronam    | 1%  | 1%  | 11%  | 6%  | 19%   |

Multi-drug resistance which was defined as resistance to 3 or more classes or sub-classes of antibiotics (Canton and Ruiz-Garbajosa, 2011), was most commonly observed in UPEC (38%) compared with commensal *E. coli* isolates (22%).

**Phylogenetic typing groups**

Phylogenetic groups A and D were commonly found among commensal *E. coli* isolates. However, UPEC isolates belonged to phylogenetic groups B2 and D, predomi-
nantly (Table 3). The results presented on Table 3 highlight a preliminary connection between pyelonephritis and phylogenetic group B2 (p < 0.001).

Distribution of hemolytic activity and hlyD in UPEC and commensal E. coli isolate

hlyD was detected in 26% of UPEC and 2% of commensal E. coli isolates, however, hemolytic activity was observed for 42% of UPEC and 6% of commensal E. coli isolates (p < 0.05).

Distribution of intI1 in UPEC and commensal E. coli isolates

intI1 gene, which was significantly associated with pyelonephritis (22%) rather than cystitis (14%) (p < 0.05), was more frequently expressed in UPEC (24%) in comparison with commensal E. coli isolates (12%).

Discussion

UTI is usually treated empirically without culture but it contributes for about 10-15% prolongation of hospitalization due to the emergence of antimicrobial resistance among the causative bacteria, particularly UPEC isolates (Walter and Stamm, 2001). This may result in the spread of antibiotic resistant bacteria in the hospital and therefore, it has been suggested that more powerful antibiotics might better eliminate UPEC reservoirs and consequently reduce the incidence of chronic and recurrent UTIs among hospitalized and outpatients (Kaper et al., 2004; Rodriguez-Siek et al., 2005).

High incidence of co-trimoxazole resistance (61% for UPECs and 82% for commensal E. coli isolates) and of susceptibility to imipenem (100% for both UPEC and commensal E. coli isolates) were detected. These data are in agreement with the results of Farshad et al. (2008) for E. coli isolates obtained from children with community-acquired UTI. Thus, co-trimoxazole, which is a widely used for UTI treatment, has become nearly ineffective to treat UTI in this country.

In our study, different antibiotic resistance patterns were observed in UPEC compared with commensal isolates. Contrarily to the results of Alhaj et al. (2007), lower resistance percentages to nalidixic acid (9%), amoxicillin (16%) and gentamicin (22%) was found among UPEC compared with commensal E. coli isolates. Nevertheless, resistance rates to ceftazidime (12%) and augmentin (14%) among UPEC isolates were in agreement with the studies of Lim et al. (2009) with 47 nonrepeat E. coli isolates, collected from intensive care unit patients presented with UTI, in 5 public hospitals located in different areas of Malaysia. Consistent with Adegoke et al. (2011), our findings revealed that cefpodoxime and cefotaxime were less effective in UTI treatment than imipenem, nalidixic acid, ciprofloxacin, nitrofurantoin, augmentin and amikacin for all UPEC phylogenetic groups.

In a research by Moreno et al. (2006), E. coli isolates obtained from 150 patients presenting with acute uncomplicated cystitis, acute pyelonephritis and urinary-source bacteraemia, revealed 21% and 18% resistance to quinolones and fluoroquinolones, respectively. Recently, Shimamura et al. (2008) has reported the emergence of fluoroquinolone resistant E. coli responsible for UTI among patients attended at Kobe University Hospital, Japan. In those studies a higher resistance to quinolones (27%) than to fluoroquinolones (5%) was observed among commensal E. coli isolates. However, they found that resistance to the two mentioned antibiotic classes was nearly the same among UPEC (9% and 8% respectively).

It should be considered that, in our study, resistance to amikacin in UPEC (8%) and commensal E. coli isolates (3%) was relatively lower, considering the 27% reported in a research conducted in Colombia by Villegas et al. (2004) on E. coli isolates obtained from hospitalized patients, in a study covering 62.3% of all general hospital beds in that country.

As previously noted, class 1 integrons were more prevalent than those of class 2 (Johnson et al., 1998; Muhammed et al., 2011; Patti et al., 2008). Similar to a research by Colgan et al. (2011), in our study intI1 gene was more frequently detected among UPEC than commensal E. coli isolates, which may contribute for the occurrence and transmission of MDR among UPEC isolates. Our results also showed that group B2 is the most frequent E. coli phylogroup in UTI, as previously found (Johnson and Russo, 2002; Kovalevskaya, 2002; Mokady et al., 2005). The UPEC isolates found in this study primarily belonged to one of two virulence groups (group B2 or D). Although a higher percentage of commensal isolates clustered into group A, a considerable proportion belonged to group D and this is

Table 3 - Phylogenetic groups distribution of UPEC and fecal E.coli strains in patients with UTI.

| Phylogenetic groups | Number of isolates | Cystitis | Pyelonephritis |
|---------------------|--------------------|----------|----------------|
| A                   |                    |          |                |
| Commensal           | 44%                | 20%      | 24%            |
| UPEC                | 8%                 | 6%       | 2%             |
| B1                  |                    |          |                |
| Commensal           | 14%                | 4%       | 10%            |
| UPEC                | 4%                 | 4%       | 0%             |
| B2                  |                    |          |                |
| Commensal           | 16%                | 0%       | 16%            |
| UPEC                | 54%                | 4%       | 50%            |
| D                   |                    |          |                |
| Commensal           | 26%                | 6%       | 20%            |
| UPEC                | 34%                | 16%      | 18%            |
why a large proportion of commensal isolates were found to represent a potential human health threat, as well as the UPEC isolates (Burman et al., 2003; Moulin-Schouleur et al., 2006).

Thus, our data indicate that group B2 *E. coli* isolates are uncommon among commensal intestinal flora (16%); however, when present, they are highly virulent (Burman et al., 2003; Moulin-Schouleur et al., 2006). In this study, only 42% of UPEC isolates had hemolytic activity, 26% of which carried hlyD gene. The relatively low percentage of hlyD gene carriage rate, in the 100 UPEC isolates analyzed here, may be partially due to the relatively low percentage of B2 isolates (54%) detected in this study. Because B2 commensal *E. coli* isolates seem to have a privileged role in eliciting urinary tract infection, the intestinal normal flora would potentially act as a reservoir for developing UTI (Branger et al., 2005). However, our findings challenge the “focal urethral” pathway for the pathogenesis of UTI in children and instead support alternative routes of infection in this population (Johnson et al., 2001a; Johnson et al., 2001b).

Many studies have shown that urine isolates collectively differed dramatically from normal flora isolates with respect to phylogenetic background and virulence gene content profiles, suggesting an increased virulence potential for the urine isolates (Clermont et al., 2000; Terai et al., 2000; Vishalakshi, 2011). In fact, in our work, a considerable difference between UPEC and commensal *E. coli* isolates was observed regarding their phylogenetic groups, presence of class 1 integron, carriage of hlyD gene, hemolysin activity and resistance pattern.

Thus, we can conclude that some UPEC with different phylogenetic characteristics and virulence profiles are multiple drug resistant (MDR) isolates which make them a serious, challenging health problem. However it is reasonable to suppose that UPEC and commensal *E. coli* isolates might have similar fitness properties for adapting to an extraintestinal lifestyle, which, in turns, enable commensal *E. coli* to cause extraintestinal disease in humans as well as UPEC. As previously mentioned, commensal *E. coli* may potentially serves as a source or reservoir of virulence genes for human pathogenesis. Further research will be necessary to determine if commensal *E. coli* isolates can actually overcome the hurdles necessary for human transmission through the urethral route.

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**Conflict of interest**

Authors have no conflict of interest.

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