

Phylogenetic Groups and Pathogenicity Island Markers in *Escherichia coli* Isolated From Children

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Received: October 14, 2012; Revised: January 11, 2013; Accepted: March 9, 2013

Background: Uropathogenic *Escherichia coli* (UPEC) is a causative agent in most of the urinary tract infections (UTIs) which expresses a multitude of virulence factors.

Objectives: In this study, we compared the presence of various PAI markers and phylogenetic groups in isolated *E. coli* from the urine of children with UTI and *E. coli* isolated from healthy children stool samples.

Materials and Methods: A total of 100 *E. coli* isolates were collected from September 2009 to August 2010. Biochemical and standard microbiological methods were used to identify the UPEC and fecal *E. coli* isolates from healthy people. All isolates subjected to phylogenetic groups typing and examination for pathogenicity islands (PAI) by multiplex PCR.

Results: We found a high number of PAI markers such as PAI ICFT073, PAI IICFT073, PAI I536, PAI IV536, PAI II J96 and PAI II536 significantly associated with UPEC. The PAI IV536 was the most detected ones among fecal *E. coli* isolated from healthy people while PAI I536, PAI II536 and PAI II96 were not seen. PCR phylogenetic groups typing revealed that uropathogenic strains were mainly found in B2 and D subgroups, while in fecal strains, A and B1 phylogenetic groups were the most common ones.

Conclusions: To our knowledge, this is the first study of the phylogenetic groups typing and PAIs marker of UPEC and fecal *E. coli* in Iran.

Keywords: Phylogenetic Typing Groups; Pathogenicity Island Markers; Uropathogenic *Escherichia coli*; Urinary Tract Infection

1. Background

Urinary tract infections (UTIs) are common in children around the world with chronic and recurrent infections resulting in a wide range of complications including end-stage kidney disease (1, 2). UTI occurs in about 3 - 5% of girls and 1% of boys, with most girls infected before 5 years; furthermore 60 - 80% of girls acquire a second infection at 18 months old after the 1st UTI (3). In adults, it is estimated that 40% of women and 12% of men will experience a symptomatic UTI, with increasing incidences in their early 20s or after age 85, respectively. About 25% of these women will infect again within 6 to 12 months.

The primary etiologic agent associated with UTIs is the strains of uropathogenic *Escherichia coli* (UPEC) (4). Although UPEC is an extracellular pathogen but can invade several types of bladder cell that compose the stratified layers of the bladder urothelium. Invasion to host cell simplifies both the establishment and persistence of UPEC in the urinary tract (5, 6). UPEC also persist inside host urothelial cells in a more quiescent state, seques-

tered within late endosomal compartments (7).

E. coli strains, present in the large intestine are categorized into 4 phylogenetic groups typing, A, B1, B2 and D; these microorganisms may be commensal, which do not harm the host or may be pathogenic, belong to groups A and B1. On the other hand, pathogenic extra intestinal *E. coli* isolates chiefly belong to phylogenetic group B2 and, to a lesser extent, group D, these isolates harbor specialized virulence factors that aid their successful colonization in the mammalian urinary tract and are responsible for intestinal and extra-intestinal disease (2, 5, 8).

Moreover, extra-intestinal *E. coli* isolates have some virulence factors which are infrequent among commensal *E. coli* isolates. Some of these virulence factors are usually encoded on pathogenicity islands (PAIs) (9). Hacker et al. first described the PAI in the late 1980s. (9, 10). These mobile genetic elements are composed of fragments of DNA (10-200 kb) adjacent to tRNA genes and contain insertion sequences, integrases and transposases, and have a G + C content that differs from the host bacterial genome.

Implication for health policy/practice/research/medical education:

Urinary tract infections (UTIs) are common in children around the world with chronic and recurrent infections resulting in a wide range of complications including end-stage kidney disease. The primary etiologic agent associated with UTIs is the strains of uropathogenic *Escherichia coli* (UPEC). In this study, we compared the presence of various PAI markers and phylogenetic groups in isolated *E. coli* from the urine of children with UTI and *E. coli* isolated from healthy children stool samples

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2. Objectives

In this study, we compared the presence of various PAI markers and phylogenetic groups in isolated *E. coli* from the urine of children with UTI and *E. coli* isolated from healthy children stool samples.

3. Materials and Methods

3.1. Specimens

This is a retrospective study from September 2009 to August 2010 on urine samples collected from 2 - 12 years old children, suspected to UTI and admitted to the nephrology ward of Mofid Children Hospital. Fresh clean catch midstream urine samples from children with UTI were collected in the sterile containers and cultured onto 5% sheep blood agar (Merck, Germany) and MacConkey agar (Merck, Germany) by means of calibrated loop delivering 0.01 mL of the urine. Plates were incubated at 37°C overnight and were examined for the growth of microorganisms. All plates showing significant growth ($> 10^5$ CFU/mL) were further processed. Also, 50 *E. coli* isolated from stool samples from healthy children were cultured on MacConkey agar plates and incubated at 37°C overnight.

Strains biochemically confirmed and the *E. coli* were stored in Luria-Bertani (LB) broth (Sigma Aldrich, Germany) supplemented with sterilized 15% glycerol (Sigma Aldrich, Germany) at -20 °C for further examination.

3.2. DNA Extraction

Three to five colonies from pure and overnight cultures were suspended in 0.5 mL sterile distilled water and lysed by heating at 95°C for 10 minutes and the supernatant was harvested by centrifugation at 8,000 rpm for 5 minutes. The supernatant was used as template DNA (11).

3.3. Phylogenetic Groups Typing

We used a simple and rapid PCR-based technique consisting of the combination of three DNA markers (*chuA*, *yjaA* and *tspE4.C2*), for phylogenetic groups typing of *E. coli* isolates (12). The expected fragment sizes were 279, 211 and 152 base pairs (bp), respectively. Multiplex-PCR procedure was performed using 3 pairs of primers in a single reaction. *E. coli* isolates fell into one of the four phylogenetic groups typing: A, B1, B2 or D. In this study the *E. coli* strain RS218 belonged to phylogenetic group B2, was used as a control in each PCR assay.

Table 1. Primer Sequences of Phylogenetic Groups Typing and Pathogenicity Island Markers

Gene	Size, bp	Primer	Reference
chuA	279	F:GACGAACCAACGGTCAGGAT, R:TGCCGCCAGTACCAAAGAC	20
yjaA	211	F:TGAAGTGTGACGAGACGCTG, R:ATGGAGAATGCGTTCCTCAA	20
TSPE4.C2	152	F:GAGTAATGTCGGGGCATTCA, R:CGCGCCAACAAAGTATTACG	20
Multiplex PCR (A) PAI III536	200	F:CGGGCATGCATCAATTATCTTG, R:TGTGTAGATGCAGTCACTCCG	2
PAI IV536	300	F:AAGGATTCGCTGTTACCGGAC, R:TCGTCCGGCAGCGTTTCTCT	2
PAI IICFT073	400	F:ATGGATGTTGTATCGCGCPS, R:ACGAGCATGTGGATCTGC	2
Multiplex PCR (B) PAI I536	1800	F:TAATGCCGGAGATTCATTGTC, R:AGGATTTGTCTCAGGGCTTT	2
PAI II 536	1000	F:CCATGTCCAAGCTCGAGC, R:CTACGTCAGGCTGGCTTTG	2
PAI I J96	400	F:TCGTGCTCAGGTCCGGAATTT, R:TGGCATCCCAATTATCG	2
PAI II J96	2300	F:GGATCCATGAAAACATGGTTAATGGG, R:GATATTTTGTGTCATTGGTTACC	2
PAI ICFT073	930	F:GGACATCCTGTACAGCACGCA, R:TGCCACCAATCACAGCCGAAC	2

The PCR mixtures were performed in 25 µL volumes containing the following: 1 µL PCR buffer (20 mM Tris-HCl, pH 8.0, 100 mM KCl, 0.1 mM EDTA, 1 M dithiothreitol, 0.5% Tween 20, 0.5% Nonidet P-40; Promega Inc., Madison, Wis.), 200 µM of each deoxynucleoside triphosphates

(New England Biolabs, Beverly, MA), 2.5 mM of MgCl₂, 2.0 U of Taq DNA polymerase (Promega Inc. USA), and primers as specified in Table 1. Crude extract (2.5 µL) was used as the template DNA for each PCR in Eppendorf Master Cycler gradient PCR system. PCR conditions were

as follow: Hot start:94°C, 4 minutes; 30 cycle (94°C in 5 seconds,59°C in 10 seconds,72°C in 10 seconds) final extension:72°C in 5 minutes (13).

3.4. Detection of PAI Markers by Multiplex PCR

All isolates were screened for the presence of PAI ICFT073, PAI IICFT073, PAI I536, PAI II536, PAI III536, PAI IV536, PAI I J96, and PAI II J96 by using two multiplex PCR. Primer sequences of PAI markers in this study are shown Table 1. PCR conditions in A and B reaction were as follow: Hot start: 94°C 5 minutes; 30 cycle (94°C in 1 minute, 55°C in 1 minute, 72°C in 1 minute) final extension: 72°C in 10 minute. The PCR products were analyzed by gel electrophoresis on 1.5% BIONEER agarose gels stained with ethidium bromide (Sigma Aldrich, Germany) (0.5 mg/mL).

3.5. Statistical Analysis

The comparisons of proportions were tested using the McNemar test. A value of P < 0.05 was considered as significant.

4. Results

4.1. Phylogenetic Groups Typing

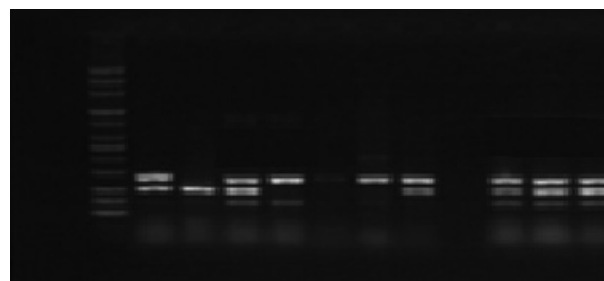
PCR results for three DNA markers (chuA, yjaA and tspE4. C2) were shown in Figure 1. The distribution of UPEC phylogenetic groups typing was as follow: 54 % B2, 34 % D, 8 % A and 4 % B1. The results showed that the distribution of B2 is more statistically significant than group D in UPEC strains (P < 0.05). In 50 fecal *E. coli* isolates, the distribution of phylogenetic groups was as follow: A; 48%, B1; 26%, D; 22 % and B2; 6%.

4.2. Pathogenicity Island Markers

In total, 89% of UPEC isolates carried the PAIs. The distribution of various PAIs showed that the most prevalent PAI were PAI IV 536 (86%) and PAI I CFT073 (74%), respectively. The prevalence of other PAIs was as follow: PAI II

CFT073 (37%), PAI II J96 (30%), PAI I 536 (36%) and PAI II536 (22%). PAI III 536 and PAI I J96 were detected only in 6% and 4% of UPEC, respectively. PAI IV 536 (18%) and PAI ICFT073 (14%) were predominant in fecal isolates. Note that some pathogenicity island markers were not seen in fecal strains. The percent of these virulence factors were more predominant in UPEC strains than fecal strains (Table 2). The PCR for detection of PAIs markers was shown in Figure 2.

Figure 1. PCR for Phylogenetic Typing (chuA, yjaA and tspE4.C2)



Lane 1: ladder, Lane 2,8: chuA, yjaA positive, Lane 3: TSPE4.C2 positive: Lane 4: chuA, yjaA, TSPE4. C2positive, Lane 5: chuA, TSPE4.C2 positive, Lane 6,9: negative control, Lane 7: chuA positive.

Figure 2. PCR for Detection of Pathogenicity Island Markers



Lane 1: ladder mid range, lane 2: PAI II 536 (1000 bp), lane 3: PAI III536 (200 bp), PAI II 536 (1000 bp), lane 4: PAI IV536 (300 bp), lane 5: PAI IICFT073 (400 bp), lane 6: PAI I536 (1800 bp), lane 7: PAI II 536 (1000 bp), lane 8: PAI I J96 (400), lane 9: PAI II J96 (2300 bp), lane 10 : PAI ICFT073 (930 bp)

Table 2. The Distribution of Pathogenicity Island Marker Genes in UPEC Isolated From Patients With UTI and Fecal *E. coli* Isolated From Health Children Stool

Pat hogeneity	UPEC (n = 50), No. (%)	Fecal <i>E. coli</i> (n = 50), No. (%)
Island genes		
PAI III536	3 (6)	1 (2)
PAI IV536	43 (86)	9 (18)
PAI IICFT073	19 (37)	3 (6)
PAI I536	18 (36)	0
PAI II 536	11 (22)	0
PAI I J96	2 (4)	0
PAI II J96	15 (30)	2 (4)
PAI ICFT073	37 (74)	7 (14)
Total	148 (37)	22 (5.5)

4.3. Distribution of PAIs According to Phylogenetic Groups in *E. coli* Isolates

The phylogenetic group B2 containing PAI IV536 was the predominant group among UPEC (Table 3). The total number of PAIs in urinary isolates belonging to phylo-

genetic groups B2 (27.75%) and D (15%) were significant. In contrast, the number of PAI in fecal isolates collected from phylogenetic group A (1.5%) and D (3%) was the highest. (Table 3) Noteworthy, there was a positive association between phylogenetic group B2 and the presence of PAIs for UPEC ($P < 0.001$).

Table 3. Distribution of Pathogenicity Islands Markers Among 50 UPEC and Fecal *E. coli* According to Phylogenetic Groups

PAI Markers	A	B1	B2	D
PAI III536				
UPEC	0	0	2	0
Fecal	0	0	1	0
PAI IV536				
UPEC	0	0	26	32
Fecal	2	1	0	6
PAI IICFT073				
UPEC	0	0	18	0
Fecal	1	0	1	1
PAI I536				
UPEC	0	0	17	2
Fecal	0	0	0	1
PAI II536				
UPEC	0	0	12	0
Fecal	0	0	0	0
PAI I J96				
UPEC	0	0	2	0
Fecal	0	0	0	0
PAI II J96				
UPEC	0	0	12	0
Fecal	1	0	1	0
PAI ICFT073				
UPEC	0	1	22	26
Fecal	2	1	0	4

5. Discussion

About 70 to 95% of community-acquired and 50% of all hospital acquired infections is caused by *E. coli*. The molecular biology details of extra intestinal *E. coli* are poorly understood. Uropathogenic *E. coli* (UPEC) can grow extra intestinally and this enables them to cause a variety of diseases. It is now demonstrated that there are a subset of fecal *E. coli* containing some virulence factors which can colonize in periurethral area, enter to the urinary tract and cause symptomatic disease such as UTI. The mentioned pathotypes are named "UPEC" (14). We studied phylogenetic and pathotypic comparison of concurrent urine and fecal *E. coli* isolates from children in 2010. We analyzed the urine samples and found that the prevalence of phylogenetic group B2 was dominant (5). In our study, *E. coli* isolates from urine samples dra-

matically differed from fecal *E. coli* isolates with regards to some virulence factors such as specific pathogenicity island markers and phylogenetic group B2 and D (15-17). Similar observation has been made by Clermont et al. in 2000 (18). According to the Herzer et al. (19) reports and our study, the pathogenicity islands were enriched in *E. coli* isolates from urinary tract infections that mostly belong to phylogenetic group B2 and D.

Dobrindt et al. (20) in 2002 studied the genetic structure and distribution of four pathogenicity islands (PAI I536 to PAI IV536) in UPEC strain 536. They also suggested that PAIs were detectable in pathogenic *E. coli* isolates than fecal ones. Our results showed that the mean number of PAIs per isolate was high in UPEC; however, *E. coli* isolates belong to groups A and B1 appeared to have fewer

pathogenicity island markers than the pathogenic isolates.

We noted that phylogenetic group B2 was uncommon in fecal isolates. This phylogenetic group is highly virulent and when colonize in intestine, may cause of UTI (2).

We can conclude that genomic differences between UPEC and fecal *E. coli* strains were mainly restricted to phylogenetic groups and large pathogenicity islands.

Acknowledgements

We thank Prof. A. Karimi and Dr. S. Maham, Dr. M. Mohkam and N. Heidari for their support.

Authors' Contribution

None declared.

Financial Disclosure

No Conflict of interest was considered in this article.

Funding/Support

This work was supported by Faculty of Medical Sciences of Tarbiat Modares University and Pediatric Infection Research Center of Mofid Children's Hospital in Tehran.

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