Arch Pediatr Infect Dis. 2017 July; 5(3):e41995. doi: 10.5812/pedinfect.41995.

Published online 2017 January 23. **Research Article**

Distribution of Uropathogenic Virulence Genes in *Escherichia coli* Isolated from Children with Urinary Tract Infection in Sanandaj, Iran

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Received 2016 September 01; **Revised** 2016 November 01; **Accepted** 2016 November 14.

Abstract

Background and Objectives: Uropathogenic *Escherichia coli* (UPEC) strains are the most common cause of urinary tract infections (UTIs) in children. UPEC isolates express a range of virulence traits promoting effective colonization of urinary tract. The aim of this study was to determine antibiotic susceptibility and virulence determinants of UPEC isolated from children.
Methods: This cross-sectional study was performed on 32 *E. coli* strains recovered from urine samples of children with UTI aged 0 to 12 years in spring 2015 (between April and June) in Sanandaj, Iran. The isolates were examined by PCR for the presence of virulence genes encoding haemolysin (*hly*), cytotoxic necrotizing factor type 1 (*cnf*1), P-fimbriae (*Pap*), and afimbrial adhesin (*afa*). Sensitivity to antibiotics was determined using the disk diffusion method.
Results: The prevalence of genes encoding adhesins was 25% for *pap*, and 15.6% for *afa*. The *hly* and *cnf* genes encoding toxins were amplified in 15.6% and 25% of isolates, respectively. The strains isolated from hospitalized patients displayed a greater number of virulence genes compared to the isolates from outpatients. Different patterns of virulence genes were identified. Nitrofurantoin and trimethoprim/sulfamethoxazole were the most and least effective antibiotics with susceptibility rates of 96.9% and 21.9%, respectively.
Conclusions: These data show the need for monitoring of drug resistance and its consideration in the treatment of *E. coli* infections. Investigation of bacterial pathogenicity associated with UTI may help have better medical intervention and management of UTI.

Keywords: Virulence Factors, Urinary Tract Infections, Drug Resistance, *Escherichia coli*

1. Background

Urinary tract infection (UTI) refers to an infection by microbial pathogens at any site of the urinary tract, which includes the urethra, bladder, ureter, and kidneys. UTI is one of the most common bacterial infections in children; many affected children, especially infants, have severe symptoms and complications (1). It is estimated that UTI is diagnosed in 1% of boys and 3% - 8% of girls (2). The most common cause of UTI in children is Uropathogenic *Escherichia coli* (UPEC). The UPEC strains harbor many virulence genes that are involved in pathogenesis of UTIs (1). There is a well-established hypothesis indicating UPEC is an evolved strain of non-pathogenic strains that has gained new virulence factors in the process of horizontal transfer of DNA (3). The UPEC strains harbor various virulence factors that contribute to the development of the infectious process, such as adhesins, toxins, serum resistance and invasion that are needed to overcome the host defense system (4).

Adhesion of *E. coli* to uroepithelial cells may protect the bacteria from washing by urine flow, increasing their ability to multiply and invade renal tissue. Pap (pyelonephritis-associated pili) and Afa (afimbrial adhesin) are the most commonly found adhesins (5). The binding of P-fimbrial adhesin to cell receptors of the renal tissue triggers specific signaling pathways leading to mucosal inflammation and tissue damage (4). Afimbrial adhesin Afa has been implicated in the development of chronic interstitial nephritis. Clinical findings suggest that UPEC strains with *Afa* adhesins have properties that favor the occurrence of recurrent and chronic UTIs (3).

Apart from adhesins, exotoxins such as α-hemolysin and cytotoxic necrotizing factor 1 (*CNF*1) are also important virulence factors. HlyA (α-haemolysin) lipoprotein is the most important secreted virulence factor of UPEC strains (6). The cytolytic effect of HlyA encoded by α-*hly* plays a role in the invasion of bacteria through the epithelial barrier (4). This toxin is able to lyse nucleated host cells for several reasons: better crossing of the mucosal barriers, having access to host nutrients and iron stores, damaging effectors immune cells, and inducing the apoptosis in T lymphocytes, neutrophils, and renal cells (6). Cytotoxic necrotizing factor 1 (CNF1) has been shown to have a role in dissemination and persistence of cells in the urinary tract. This

toxin causes bladder cell exfoliation and increases bacterial access to the underlying tissue (4).

Urinary tract infections, which are non-properly managed from their onset, can become a real threat ultimately leading to renal failure. Drug-resistant UPEC strains, which are increasing in prevalence worldwide, may cause significant deleterious impacts on the clinical management of UTI (1).

2. Objectives

A few studies have examined the virulence properties and antibiotic resistance of UPEC isolated from children in Sanandaj, Iran. Therefore, in this study, we sought to identify the prevalence and expression patterns of the most important virulence genes involved in the development of UTI including *pap*, *afa*, *hly*, and *cnf*1 (4, 6-8). Also, we aimed to evaluate antimicrobial resistance among *E. coli* strains isolated from children with UTIs in Sanandaj. The study gives insights into the current antibiotic resistance pattern and virulence properties in UPEC isolated from children in this part of the country.

3. Methods

3.1. Bacterial Isolates and Identification

In this cross-sectional study, 32 non-duplicate consecutive UPEC strains were isolated in spring 2015 (between April and June) from children with UTIs aged 0 - 12 years admitted to Besat and Tohid tertiary hospitals in Sanandaj, Iran. Sanandaj is the center of Kurdistan province in the west of Iran with a population of more than 480,000. Tohid and Besat are two referral and teaching general hospitals that are affiliated to Kurdistan University of Medical Sciences. Any case with positive urine culture of $\geq 10^5$ colony-forming units (cfu)/mL was considered as UTI. The *E. coli* isolates were identified based on various standard bacteriological and biochemical methods such as Gram staining, typical morphology, lactose fermentation, production of gas, indole test, citrate utilization, motility, lysine decarboxylation, methyl red, Voges-Proskauer, etc. (9). All bacterial isolates were stored at -70°C in tryptic soy broth (Quelab, New Mexico, U.S.) containing 15% glycerol.

3.2. Antibiotic Susceptibility Test

The susceptibility of bacterial strains to antibiotics were determined using disk diffusion method conducted on Mueller-Hinton agar plates (Merck, Germany) as per the 2014 guidelines of clinical and laboratory standards institute (CLSI) (10). The disks used in the susceptibility test contained the following antibiotics (Rosco company, Denmark): ampicillin (10 μg), cefotaxime (30 μg), ceftazidime (30 μg), imipenem (10 μg), amoxicillin/clavulanic acid (30 μg), aztreonam (30 μg), ciprofloxacin (5μg), tetracycline (30 μg), trimethoprim/sulfamethoxazole, gentamicin (10 μg), cefepime (30 μg), cefoxitin (30 μg), amikacin (30 μg), norfloxacin (10 μg), nalidixic acid (30 μg), and nitrofurantoin (300 μg). The susceptibility of isolates to each antimicrobial agent was measured. *E. coli* ATCC 25922 was used as quality control.

3.3. DNA Extraction

Genomic DNA was prepared by the freeze-thaw method and used as the template for PCR (11). *E. coli* strains were grown in brain-heart infusion (BHI) broth (Quelab, New Mexico, U.S.) at 37°C overnight. The bacterial cells were then pelleted from 1 mL BHI broth and suspended in 200 μL of sterile distilled water, followed by incubation at 100°C for 10 minutes. The suspensions were immediately placed on ice for 5 minutes. Samples taken through a total of 3 freeze-thaw cycles were centrifuged and the supernatants were stored at -20°C as template DNA stocks.

3.4. Detection of Virulence Genes

Specific primers (SinaClon, Iran) were used to amplify sequences of the *pap*, *afa*, *hly*, and *cnf*-1 genes. Details of primer sequences and predicted sizes of the amplified products are shown in Table 1. Amplification of bacterial DNA was done in a total volume of 25 μL containing 3 μL DNA extract, 0.4 μM of each primer, 1X PCR buffer, 1.5 mM $MgCl_2$, 200 μM of each dNTP, and 1 U Taq DNA polymerase (SinaClon, Iran). The amplification was performed in a thermal cycler (Eppendorf, Germany) under the following conditions: Initial denaturation at 94°C for 5 minutes; then 35 cycles of denaturation at 94°C for 1 minutes, annealing at 65°C for 1 minutes, and extension at 72°C for 1 minutes followed by a final elongation at 72°C for 7 minutes. Conditions were the same for all genes. The PCR products were electrophoresed in a 1% agarose gel (SinaClon, Iran) in 0.5X TBE (Tris-Borate-EDTA) buffer, stained with DNA safe stain (SinaClon, Iran), and photographed using a UV transillumination imaging system. The size of the amplicons was estimated by comparing them to a 100 bp Plus DNA ladder (SinaClon, Iran). The positive strains were kindly provided by Dr. S. Najar Peerayeh (Tarbiat Modares University, Tehran) and Dr. A. Rashki (University of Zabol, Zabol).

3.5. Statistical Analysis

All statistical analyses were performed using SPSS version 16.0 (SPSS Inc., Chicago, USA). Data were analyzed us-

Table 1. Sequence of Primers Used to Detect Virulence Genes and Predicted Sizes of the Amplified Products of PCR

Target	Primer Name	Primer Sequence (5' - 3')	Size of Amplicons, bp	Reference
pap	*Pap*1	GACGGCTGTACTGCAGGGTGTGGCG	328	(12)
	*Pap*2	ATATCCTTTCTGCAGGGATGCAATA		
afa	*afa*1	GCTGGGCAGCAAACTGATAACTCTC	750	(12)
	*afa*2	CATCAAGCTGTTTGTTCGTCCGCCG		
hly	*hly*1	AACAAGGATAAGCACTGTTCTGGCT	1177	(13)
	*hly*2	ACCATATAAGCGGTCATTCCCGTCA		
cnf	*cnf*1	AAGATGGAGTTTCCTATGCAGGAG	498	(13)
	*cnf*2	CATTCAGAGTCCTGCCCTCATTATT		

ing Pearson's Chi-square test and Fisher's exact test. A P value < 0.05 was considered statistically significant.

4. Results

A total of 32 UPEC strains were collected from children with UTI aged 0 - 12 years between April and June 2015. The male to female ratio in the study group was approximately 1:2 (10 males and 22 females) and the mean age was approximately 6 years. Twenty-one out of 32 patients were hospitalized while 11 were outpatients.

A total of 19 (59.4%) isolates were found to harbor at least one of the four virulence genes investigated. With regard to adhesion virulence determinants, the *pap* gene was present in 25% (8) and *afa* in 15.6% (5) of the 32 isolates. Among the genes encoding toxins, *hly* was found in 15.6% (5), while *cnf* was present in 25% (8) of the 32 isolates. Thirteen strains were negative for all the studied virulence genes. The *hly* and *pap* gens were found more frequently in females than males (18.2% vs. 10%; 27.3% vs. 20%, respectively). By contrast, females expressed a lower prevalence of *cnf* and *afa* than males (18.2% vs. 40%; 13.6% vs. 20%, respectively). However, the prevalence of virulence genes was not significantly different based on sex group (P > 0.05).

With the exception of *afa* gene that was detected more prevalently in the outpatient group than the inpatient group (4/11, 36.4% vs. 1/21, 4.8%), the prevalence of other virulence genes was higher in the inpatient group. The *cnf*, *hly*, and *pap* genes were present in 28.6%, 19%, and 28.6% of the 21 strains isolated from inpatients, respectively; while they were found in 18.2%, 9.1% and 18.2% of the 11 strains collected from outpatients, respectively. No statistically significant difference was seen between the two groups concerning the prevalence of virulence factors (P > 0.05), except for *afa* that was more expressed in outpatients (P = 0.019).

The isolated strains were classified in eight patterns of virulence gene expression according to various targeted sequences. The patterns are referred to as Ec followed by an Arabic numeral (Table 2). The Ec1 pattern that lacked the studied virulence genes was the most commonly detected pattern (found in 13 isolates), followed by Ec7 (found in 7 isolates) that was characterized by the presence of *cnf* gene only. The Ec3, Ec4, and Ec6 patterns were detected only in the hospitalized patients. The Ec2 profile which was detected only in the outpatient group and characterized by the presence of *afa* gene showed a meaningful distribution between the in- and out-patient groups (P = 0.012). The maximum number of detected amplicons in one strain was three of the targeted virulence gene regions. A significant association was detected between the simultaneous presence of *hly* and *pap* virulence genes (P = 0.002), which corresponded to 12.5% of the UPEC strains. Two isolates carried both *pap* and *afa* genes, and one isolate carried both *pap* and *cnf* genes, although no significance was observed according to Chi-square and Fisher's tests.

Antibiotic susceptibility of the UPEC strains was carried out using different classes of antibiotics. Of the 32 isolates, 96.9% were susceptible to nitrofurantoin (n = 31), 93.8% to imipenem (n = 30), and 90.6% to cefoxitin (n = 29). The least effective antibiotics were cefotaxime, ampicillin, and trimethoprim/sulfamethoxazole, which gave the susceptibility rates of 34.4%, 28.1%, and 21.9%, respectively (Table 3). Isolates from inpatients were more susceptible to cefotaxime, ceftazidime, cefepime, tetracycline, amoxicillin/clavulanic acid, imipenem, norfloxacin, nalidixic acid, and ciprofloxacin compared to the isolates from outpatients (Table 3).

There was no relationship between the presence of virulence genes and antimicrobial susceptibility of UPEC, except for *cnf* gene and nalidixic acid (P < 0.05). The *cnf* gene was more prevalent in nalidixic acid-susceptible isolates than non-susceptible isolates (resistant plus intermediate

Table 2. Virulence Patterns Identified Among 32 Uropathogenic *Escherichia coli* Isolated from Children

Pattern	Virulence Gene				No. of Positive Strains in Hospitalized Patients	No. of Positive Strains in Outpatients	Total No. of Strains
	hly	*afa*	*pap*	*cnf*			
Ec1	-	-	-	-	9	4	13
Ec2	-	+	-	-	0	3	3
Ec3	+	-	-	-	1	0	1
Ec4	+	-	+	+	1	0	1
Ec5	+	-	+	-	2	1	3
Ec6	-	-	+	-	2	0	2
Ec7	-	-	-	+	5	2	7
Ec8	-	+	+	-	1	1	2

Table 3. Antimicrobial Susceptibility of 32 Uropathogenic *Escherichia coli* Isolated from in- and Out-Patient Groups[a]

Antibiotic	Susceptibility		
	Inpatients (n = 21)	Outpatients (n = 11)	Total (n = 32)
Nitrofurantoin	20 (95.2)	11 (100)	31 (96.9)
Imipenem	21 (100)	9 (81.8)	30 (93.8)
Cefoxitin	19 (90.5)	10 (90.9)	29 (90.6)
Amoxicillin/clavul acid	19 (90.5)	9 (81.8)	28 (87.5)
Amikacin	18 (85.7)	10 (90.9)	28 (87.5)
Norfloxacin	16 (76.2)	6 (54.5)	22 (68.8)
Gentamicin	13 (61.9)	7 (63.6)	20 (62.5)
Ceftazidime	13 (61.9)	6 (54.5)	19 (59.4)
Aztreonam	12 (57.1)	7 (63.6)	19 (59.4)
Cefepime	12 (57.1)	5 (45.5)	17 (53.1)
Ciprofloxacin	14 (66.7)	4 (36.4)	18 (56.3)
Tetracycline	8 (38.1)	4 (36.4)	12 (37.5)
Nalidixic acid	10 (47.6)	2 (18.2)	12 (37.5)
Cefotaxime	8 (38.1)	3 (27.3)	11 (34.4)
Ampicillin	5 (23.8)	4 (36.4)	9 (28.1)
Trimethoprim/sul	4 (19)	3 (27.3)	7 (21.9)

[a]Values are expressed as No. (%).

(for cefepime: Susceptible-Dose Dependent, SDD)) (50% vs. 10%, $P < 0.05$).

5. Discussion

E. coli causes the vast majority of UTI in both outpatients and inpatients. The degree of severity of infection depends on the virulence of the responsible strains (3). The most important virulence factors in UPEC strains are P-fimbriae (*pap*), afimbrial adhesin (*afa*), hemolysin (*hly*), and cytotoxic necrotizing factor 1 (*cnf1*) (4, 6-8). The ability of bacteria to attach to uroepithelial cells through adhesins is critical for the initiation of infection (14). Of the 32 uropathogenic *E. coli* isolates tested by PCR in the present study, 25% and 15.6% exhibited *pap* and *afa* adhesins, respectively. Previous studies have shown the prevalence of *pap* gene in UPEC strains isolated from children as 70% and 30.2% in two Iranian studies (15, 16), 39.6% in a South Korean study (4), and 22.9% in a Turkish study (17). The prevalence of *afa* was 9.4% in Yun et al.'s study (1), 26.6% in Dormanesh et al.'s study (15), and 66.6% in Tajbakhsh et al.'s study (18), which are different from the rates found in our study.

Toxins are important virulence factors mediating invasion, dissemination, and persistence of bacteria in host cells (4). The toxins, α-hemolysin and CNF1, are believed to act by release of iron from red blood cells, dysfunction of phagocytic cells, and direct cytotoxicity to the tissues (14). Haemolysin is needed for the initial invasion of bacteria through the epithelial barrier, while CNF1 is needed for the dissemination and persistence of *E. coli* strains (4). *hly* and *cnf*1 genes were present in 15.6% and 25% of our isolates, respectively. The distribution of the *hly* gene among the studied isolates was lower than that previously reported (1, 19, 20) although it is in agreement with those reported by Farshad et al. (16) and Alizadeh et al. (21) in Iran. The prevalence of *cnf-1* was 65.5% among Korean children (4), 56.66% in Iranian children (15), 27% in children hospitalized in Australia (20), and 9% in a Pakistani population (22). We found that *E. coli* strains obtained from hospitalized patients carried more virulence genes and hence, they are appeared to be more aggressive than the strains isolated from outpatients. This finding is in accordance with the

results of previous studies (3, 23). The *afa* virulence gene was more prevalent in outpatients, which emphasizes the importance of admission of these infected patients to tertiary care teaching hospitals. Virulence factors may have distinctive, complex associations with one another (1). Our results revealed the occurrence of specific gene combination as *pap-hly*, which corresponded to 12.5% of the UPEC strains ($P < 0.05$) (Table 2). Recently, Regua-Mangia et al. have demonstrated the occurrence of specific gene combinations as *pap-afa* and *pap-cnf* (24). Birosova et al. also observed that *afa* gene was associated with *pap* sequence (25). In our study, two isolates carried both *pap* and *afa* genes, and one isolate carried both *pap* and *cnf* genes, although they showed insignificant associations according to Chi-square and Fisher's tests.

Early diagnosis and prompt antibacterial treatment are critical to minimize renal scarring and progressive kidney damage in patients with UTI. Several studies have reported a relationship between antimicrobial resistance and virulence factors in UPEC. For example, tetracycline resistance has been associated with a higher prevalence of *pap* ($P < 0.05$) (1), and a lower prevalence of *pap*, *cnf*1, and *hly* has been reported in fluoroquinolone-resistant strains in comparison with their susceptible counterparts (26). In our study, an association was seen between the susceptibility to nalidixic acid and presence of *cnf* gene ($P < 0.05$). The presence of certain virulence genes might be dependent on the mechanisms of antibiotic action or an unknown interaction between virulence factors and antibiotics. Nitrofurantoin and imipenem antibiotics showed the highest activity against the isolates, which is in agreement with the results of other reports (27, 28). It seems that in our study, isolates from outpatients were more resistant to the tested antibiotics in comparison with isolates from inpatients, which highlights the problem of admission of these infected patients to hospital. Although most antibiotic-resistant bacteria have originally emerged in hospitals, drug-resistant strains have been increasing in the community, worldwide (29). The development of resistance in the community might be due to inappropriate use of antibiotics, the continued use of antibiotics in agriculture and animals, and ineffective infection control and health programs (30). It is estimated that 80% - 90% of human antimicrobial drugs are taken by outpatients and the remained 10% - 20% by hospitalized patients. Also, 20% - 50% of antibiotics are believed to be consumed uncertainly (31). These may expose human population to the increased risk of side effects, higher economic burden, and more resistant pathogens to antibiotic compounds.

In conclusion, this study highlights the distribution of virulence factors and the antibiotic resistance among UPEC isolated from children in Sanandaj. A better knowledge on the antibiotic resistance and virulence properties of microorganisms causing the infection may help clinicians predict the evolution of infection in the host.

Acknowledgments

We are grateful to the staff of the microbiology laboratories at Besat and Tohid hospitals for collecting *E. coli* isolates used in this study.

Footnotes

Author Contribution: Maryam Pourzare performed the microbiological and molecular studies. Safoura Derakhshan designed the study and prepared the manuscript. Daem Roshani advised the study.

Financial Disclosure: There is no financial interest related to the materials in the manuscript.

Funding/Support: This study was supported by Faculty of Medicine, Kurdistan University of Medical Sciences, Sanandaj, Iran.

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