International Journal of Infectious Diseases 29 (2014) 219–222

Contents lists available at ScienceDirect

International Journal of Infectious Diseases

journal homepage: www.elsevier.com/locate/ijid

Detection of virulence genes in *Escherichia coli* isolated from patients with cystitis and pyelonephritis

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ARTICLE INFO

Article history:
Received 21 January 2014
Received in revised form 8 March 2014
Accepted 27 March 2014

Corresponding Editor: Eskild Petersen, Aarhus, Denmark

Keywords:
Uropathogenic *Escherichia coli*
Pyelonephritis
Cystitis
Virulence genes

SUMMARY

Background: Uropathogenic *Escherichia coli* (UPEC) is a common cause of ascending urinary tract infections including cystitis and pyelonephritis. The purpose of this study was to investigate virulence genes among *Escherichia coli* isolated from patients with cystitis and pyelonephritis.
Methods: Between December 2012 and June 2013, 150 *E. coli* isolates from hospitalized patients with pyelonephritis (n = 72) and cystitis (n = 78) were collected at Shahid Beheshti Hospital in Kashan. A PCR assay was used to evaluate the presence of virulence genes including *pap*, *hly*, *aer*, *sfa*, *cnf*, *afa*, *traT*, and pathogenicity island (PAI) markers in isolates.
Results: Of the total 150 UPEC isolates, 130 (86.7%) were found to carry the virulence genes studied. Nineteen different virulence patterns were identified. The most prevalent virulence pattern was UPEC including *traT*–PAI operons. The *pap*, *traT*, *aer*, *hly*, and PAI operons were more prevalent among patients with pyelonephritis than cystitis, and the *sfa*, *afa*, and *cnf* genes were not detected in any of the isolates.
Conclusions: Higher virulence gene diversity was found among pyelonephritis UPEC isolates in comparison to cystitis UPEC isolates, showing that UPEC strains that cause pyelonephritis need more virulence factors.

1. Introduction

Uropathogenic *Escherichia coli* (UPEC) is the major causative agent of urinary tract infections (UTI) including cystitis and pyelonephritis.[1] In ascending infections, colonization of fecal organisms in the urethra leads to the upward spread of bacteria to the bladder (causing cystitis) and kidneys (causing pyelonephritis).[2,3]

UPEC contains several virulence factors that facilitate its colonization and invasion of host cells.[4,5] Surface virulence factors (adhesins) of UPEC are among the most important virulence factors.[1,6] As the main attachment factor, P fimbriae is particularly associated with pyelonephritis and is encoded by *pap* genes.[7] Another adhesion that acts as a virulence factor is S fimbrial adhesion, which is coded by *sfa* genes.[1,8] Also, afimbrial adhesions (Afa) of *E. coli*, coded by *afa* genes, have been reported in cases of pyelonephritis and recurring cystitis.[9]

Other important virulence factors of UPEC strains are the toxins that act as secretory virulence factors.[1] The most important secretory virulence factor is α-hemolysin (HlyA), which is encoded by the *hly* gene. Also, cytotoxic necrotizing factor 1 (CNF1) is reported in a third of pyelonephrogenic strains.[10] Other virulence factors also have important roles in the development of UTIs, including serum resistance ability due to the outer membrane protein TraT encoded by *traT* genes.[11,12] Aerobactin is a bacterial siderophore encoded by *aer* genes and has recently been documented as a virulence factor in UPEC strains.[13] The virulence factors are carried by pathogenicity islands (PAIs), which are mobile genetic elements that contribute to the horizontal transfer of virulence determinants.[14,15]

The characterization of virulence genes can be useful to improve our understanding of the pathogenesis of UTI and to minimize the complications, including kidney failure. Few studies have sought to detect the prevalence of virulence genes in UPEC strains causing distinct types of UTIs in our region. Therefore the present study was proposed to determine the prevalence of virulence genes among UPEC strains isolated from patients with cystitis and pyelonephritis.

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http://dx.doi.org/10.1016/j.ijid.2014.03.1393

2. Materials and methods

2.1. Survey area

The city of Kashan, with a population of approximately 400 000 people, is the most populated city in the north of Isfahan Province in the center of Iran. It is located at an altitude of 1600 m above sea level and has the geographical coordinates 50° 38′ 00″, 36° 13′ 00″.

2.2. Bacterial isolates

One-hundred and fifty *E. coli* strains, including 72 isolates from patients with pyelonephritis and 78 isolates from patients with cystitis, were collected during December 2012 to June 2013. *E. coli* strains were isolated from patients of both sexes (78% female, 22% male) aged between 1 and 95 years, with a mean age of 50 years.

UTI was determined by positive urine culture (10^5 colony-forming units/ml) and the presence of 10^4 leukocytes/ml of urine indicating pyuria. UPEC was divided into two groups: (1) UPEC strains related to pyelonephritis, and (2) UPEC strains related to cystitis. Cystitis was characterized by typical clinical symptoms including dysuria, frequent voiding, and lower abdominal pain, whereas pyelonephritis was identified clinically by fever, nausea, dysuria, urgent voiding, flank pain, and lumbar tenderness. Standard biochemical tests were used for the isolation and identification of *E. coli* strains.[16]

2.3. DNA extraction

DNA extraction was performed using the boiling method. Before DNA extraction, the *E. coli* isolates were cultured in LB broth at 37 °C for 18 h. Bacteria were pelleted from 1.5 ml LB broth then suspended in 200 μl of sterile deionized water and incubated at 100 °C for 10 min. After centrifuging, the supernatant was used as template DNA and stored at −20 °C.

2.4. PCR amplification of virulence genes

PCR assays were used to reveal the prevalence of eight virulence genes including *pap*, *sfa*, *afa*, *hly*, *cnf*, *aer*, *traT*, and PAI markers using specific primers.[17–19]

The PCR was performed in a mixture consisting of 5 μl of DNA, 1 μl (10 pmol) of each of the primers, 200 μM of dNTP, 1.25 U Taq DNA polymerase, and 2.5 μl of 10× reaction buffer containing 1.5 mM $MgCl_2$. The amplification of virulence genes was carried out in a Thermal Cycler (Eppendorf Master Cycler) and the PCR conditions were the following: an initial denaturation at 94 °C for 2 min, followed by 30 cycles of denaturation at 94 °C for 60 s, annealing at 63 °C for 30 s, and extension at 72 °C for 90 s, with a final extension at 72 °C for 5 min.

The electrophoresis of PCR products was performed on 1.8% agarose gels with a 100-bp DNA ladder as molecular size marker (100 bp DNA ladder; MBI Fermentas). The gels were stained with ethidium bromide for 15 min and visualized in a gel document system (Bio-Rad, UK).

2.5. Statistical analysis

The statistical analysis was performed using SPSS software version 15 (SPSS Inc., Chicago, IL, USA). The Chi-square test was used to evaluate correlations of the variables. *p*-Values less than 0.05 were considered significant.

Table 1
Prevalence of virulence genes among UPEC isolates causing pyelonephritis and cystitis

Virulence gene[a]	No. of pyelonephritis isolates (%) (n = 72)	No. of cystitis isolates (%) (n = 78)	*p*-Value
PAI	59 (81.9)	33 (42.3)	<0.001
pap	20 (27.8)	5 (6.4)	<0.001
traT	58 (80.6)	53 (67.9)	0.07
aer	27 (37.5)	19 (24.4)	0.08
sfa	0 (0)	0 (0)	-
afa	0 (0)	0 (0)	-
hly	5 (6.9)	1 (1.3)	0.1
cnf	0 (0)	0 (0)	-

UPEC, uropathogenic *Escherichia coli*.
[a] PAI, pathogenicity islands; *pap*, pilus associated with pyelonephritis; *traT*, serum resistance associated gene; *aer*, aerobactin; *sfa*, S fimbrial adhesion; *afa*, afimbrial adhesions; *hly*, α-hemolysin; *cnf*, cytotoxic necrotizing factor 1.

3. Results

The prevalence of virulence genes in patients with pyelonephritis and cystitis is shown in Table 1. In total, 130/150 (86.7%) UPEC strains contained the studied virulence genes, of which 34 (22.6%), 52 (34.6%), 39 (26%), and 5 (3.5%) were found to carry one, two, three, and four virulence genes, respectively.

Nineteen different virulence patterns were identified among UPEC strains (Table 2). The pyelonephritis and cystitis isolates showed 17 and 12 virulence patterns, respectively (Table 3). UPEC6 was determined by the presence of the *traT* and PAI genes, and was the most identified pattern, found in 37 (24.6%) isolates. *traT* was the most common virulence gene and was detected in 58 (80.6%) UPEC isolates from pyelonephritis and 53 (67.9%) UPEC isolates from cystitis. Among the adhesion virulence genes, *pap* was the most prevalent gene and was identified in 25 (16.7%) isolates, while *sfa* and *afa* genes were not detected in any of the isolates. The secretory virulence genes including *hly* and *cnf* were present in six (4%) and none of the UPEC isolates, respectively. The *aer* gene was identified in 46 (30.7%) and the PAI markers were detected in 92 (61.3%) of the isolates. The statistical analysis showed significant differences

Table 2
Different virulence gene patterns among the studied UPEC strains

Virulence pattern	Virulence gene[a]								No. (%) of strains
	PAI	*pap*	*traT*	*aer*	*sfa*	*afa*	*hly*	*cnf*	
UPEC1	–	–	–	–	–	–	–	–	20 (13.3)
UPEC2	–	–	+	–	–	–	–	–	22 (14.6)
UPEC3	+	–	–	–	–	–	–	–	6 (4)
UPEC4	–	–	–	+	–	–	–	–	5 (3.3)
UPEC5	–	+	–	–	–	–	–	–	1 (0.7)
UPEC6	+	–	+	–	–	–	–	–	37 (24.6)
UPEC7	–	–	+	+	–	–	–	–	6 (4)
UPEC8	–	+	+	–	–	–	–	–	1 (0.7)
UPEC9	–	–	+	–	–	–	+	–	1 (0.7)
UPEC10	+	–	–	+	–	–	–	–	3 (2)
UPEC11	+	+	–	–	–	–	–	–	3 (2)
UPEC12	+	–	+	+	–	–	–	–	22 (14.7)
UPEC13	+	+	+	–	–	–	–	–	10 (6.7)
UPEC14	+	–	+	–	–	–	+	–	2 (1.3)
UPEC15	–	+	+	+	–	–	–	–	3 (2)
UPEC16	+	+	–	+	–	–	–	–	3 (2)
UPEC17	+	+	+	+	–	–	–	–	3 (2)
UPEC18	+	–	+	+	–	–	+	–	1 (0.7)
UPEC19	+	+	+	–	–	–	+	–	1 (0.7)

UPEC, uropathogenic *Escherichia coli*.
[a] PAI, pathogenicity islands; *pap*, pilus associated with pyelonephritis; *traT*, serum resistance associated gene; *aer*, aerobactin; *sfa*, S fimbrial adhesion; *afa*, afimbrial adhesions; *hly*, α-hemolysin; *cnf*, cytotoxic necrotizing factor 1.

Table 3
Distribution of virulence gene pattern in relation to UTI type

Virulence gene pattern	Virulence pattern, No.		Total
	Pyelonephritis	Cystitis	
UPEC1	2	18	20
UPEC2	5	17	22
UPEC3	3	3	6
UPEC4	2	3	5
UPEC5	0	1	1
UPEC6	21	16	37
UPEC7	2	4	6
UPEC8	1	0	1
UPEC9	0	1	1
UPEC10	3	0	3
UPEC11	2	1	3
UPEC12	12	10	22
UPEC13	8	2	10
UPEC14	2	0	2
UPEC15	2	1	3
UPEC16	3	0	3
UPEC17	3	0	3
UPEC18	1	0	1
UPEC19	1	0	1

UTI, urinary tract infection; UPEC, uropathogenic *Escherichia coli*.

($p < 0.001$) in the presence of the *pap* gene and of PAI markers between patients with pyelonephritis and cystitis (Table 1).

4. Discussion

UTI caused by UPEC is one of the most prevalent infectious diseases leading to renal failure.[20] The degree of pathogenicity of UPEC strains is dependent on the presence of virulence factors.[21]

Fimbriae-associated adherence is an important factor in the pathogenesis of UTI, and the critical role of P fimbriae in the development of pyelonephritis is well known.[17,22] Our results showed that the frequency of the *pap* gene in pyelonephritis and cystitis strains was 27.8% and 6.4%, respectively. Our findings are in accordance with the results reported by Mabbett et al., showing the *pap* gene to be significantly more prevalent among patients with pyelonephritis than cystitis.[23] Of note, other adhesion - associated virulence determinants including *sfa* and *afa* genes were not found among the UPEC isolates in our study. In a similar study conducted by Qin et al.[24], it was shown that none of the UPEC isolates from the pyelonephritis and cystitis groups carried the *afa* gene. Also, the results of a survey in China showed that *sfa* was not detected in pyelonephritis UPEC strains.[25] The strains that lack S fimbriae and afimbrial adhesion perhaps use a different adhesion such as P fimbriae for attachment. Our results showed a higher prevalence of the *hly* (α-hemolysin) gene among UPEC isolates causing pyelonephritis (6.9%) in comparison to isolates causing cystitis (1.3%). Another toxin-associated virulence gene, *cnf*, was not identified in our UPEC strains. The prevalence of virulence genes coding for toxins have been documented at between 0 and 44% and between 0 and 30% for *hly* and *cnf* operons, respectively.[19] These findings show that the prevalence of these genes varies by geographical region.

traT, the serum resistance-associated gene, was the most prevalent virulence determinant. Oliveira et al.,[19] also in agreement with our findings, reported the *traT* gene to be the most prevalent virulence gene among UPEC strains. These results show that *traT* is a common and important virulence factor and could be considered as a target for therapeutic interventions.

In our study, the presence of PAI markers and the frequency of the *aer* gene were significantly higher among patients with pyelonephritis than patients with cystitis ($p < 0.001$). It has been accepted that the PAIs, as mobile genetic elements, are capable of horizontal gene transfer between species, so a frequency of 81.9% for PAI markers in strains isolated from patients with pyelonephritis is considerable.

The association among the different virulence factors in UPEC isolates has been documented.[21] Nineteen different virulence patterns were found among the UPEC strains regarding the frequency of virulence determinants. Also the strains isolated from patients with pyelonephritis demonstrated more virulence gene diversity than strains isolated from patients with cystitis. Similar results were documented by Olivera et al.[19] The most prevalent virulence pattern was *traT*–PAI, which was detected in 24.6% of UPEC isolates. In Brazil, the prevalence of the *traT*–PAI virulence gene pattern was determined to be 3.92%.[17] Also the percentages of the virulence gene patterns including *traT* and *traT*–PAI–*aer* were equal and were found to be the second most frequent patterns in our study. In contrast, *traT* was reported as the most common virulence pattern by Olivera et al.[19] The association of the *pap* operon with other adhesin-encoding operons (*sfa* and *afa*) was not detected in our study. Bouguenec et al.[26] did not detect the simultaneous presence of *pap*, *sfa*, and *afa* operons among UPEC strains.

In conclusion, in our patients, we found that pyelonephritis-producing strains of *E. coli* had significantly more virulence factors than did cystitis-producing strains, raising the possibility that this increase in virulence factors may lead to an increased risk of pyelonephritis.

Acknowledgements

The technical assistance of Mr Pourbabaei is acknowledged with pleasure. The critical comments of Professor Moniri of the Department of Microbiology and Immunology, School of Medicine, Kashan University of Medical Sciences is gratefully acknowledged. The results described in this paper formed part of an MSc student thesis (Foroogh Neamati).

Conflict of interest: The authors declare no funding or potential conflicts of interest relevant to this article.

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