Frequency distribution of virulence factors in uropathogenic *Escherichia coli* isolated from Kermanshah in 2011-2012

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Abstract

Background: Uropathogenic *Escherichia coli* (UPEC) can cause urinary tract infection (UTI). To prevent urine flow lavage, UPEC has acquired several virulence factors called adhesins. These adhesins are expressed and controlled by different genes. **Aim:** This study was aimed to determine some of the most important genes that control virulence factors of UPEC (pyelonephritis associated pili [*pap*], S fimbrial adhesion [*sfa*] and A fimbrial adhesion [*afa*] genes), which code for adhesins and phenotypic factors. **Materials and Methods:** In total, 205 UPEC isolates from in- and out-patients with UTI were obtained. Polymerase chain reaction was used for gene amplification. One drop of bacterial suspension, one of red blood cells and one of peripheral blood smear were mixed for hemagglutination (HA). Formation of a clump was considered to be positive. Bacteria were grown on blood agar to determine hemolysis. Surface hydrophobicity was determined using the SAT test. **Result:** Frequencies of *pap*, *afa* and *sfa* were 42 (20.5%), 17 (8.3%) and 44 (21.5%), respectively. Frequencies of HA, hemolysis and hydrophobicity were 138 (67.3%), 56 (27.3%) and 39 (19%), respectively. Among HA-positive bacteria, 103 (74.6%) were mannose resistant. Our results highlight higher frequency of HA than that of other virulence factors, indicating a crucial role of this virulence factor in UPEC. **Discussion:** We concluded that major differences exist in the prevalence of virulence factors among different UPEC isolated from different countries. The association observed between pathogenicity and virulence factors may promote UPEC survival and growth within the urinary tract. Detecting these genes as the primary controllers of UPEC virulence factors may aid in better management of related infections.

Key words: Hemagglutination, hemolysin, hydrophobicity, uropathogenic *Escherichia coli*, virulence factors

Introduction

Urinary tract infection (UTI) is a prevalent infectious condition with potentially severe complications, thus representing a major cause of human discomfort.[1] UTIs are one of the most frequently encountered infections in developing countries as well as one of the most common medical problems. It is estimated that 150 million patients are diagnosed with UTI every year, which result in at least $6 billion in health care expenditures.[2] Clinical manifestations of UTI are highly variable in extent and severity, with cystitis generally resolving without sequela, whereas pyelonephritis can cause serious morbidity and fatality.[3]

A broad spectrum of bacteria can cause UTI, such as *Klebsiella*, *Proteus*, *Enterobacter*, *Pseudomonas*, *Serratia* and *Citrobacter* species. *Escherichia coli* is by far the most common cause of UTI, accounting for 80-90% of all UTIs among ambulatory populations.[4] *E. coli* isolates are divided to intestinal, extra intestinal and commensal pathogens[5] and the varitypes causing UTI are called uropathogenic *E. coli* (UPEC). UPEC is the most common etiological agent of community-acquired UTIs, accounting for more than 80% of these infections.[6]

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The severity of the infection depends on both the virulence of the infecting bacteria and the susceptibility of the host. UPEC adhesions to the uroepithelium may protect the bacteria from urinary lavage, thus increasing their ability to multiply and invade the renal tissue. UPEC strains encode several virulence factors, which enable the bacteria to colonize the urinary tract and persist in the presence of highly effective host defense. UPEC possess adherence factors called pilus or fimbriae, which allow them to successfully initiate infections. The 2 primary pili in UPEC are type 1 and P. Moreover, specific adhesion is mediated by bacterial proteins called adhesions, which may or may not be associated with fimbriae, e.g., pyelonephritis associated pili (*pap*), S fimbrial adhesion (*sfa*) and A fimbrial adhesion (*afa*).[7] In the late 1970s, it was recognized for the first time that UTI-causing *E. coli* strains typically agglutinate human erythrocytes despite the presence of mannose (mannose-resistant hemagglutination [MRHA]) and adhere to human uroepithelial cells. Moreover, adherence to uroepithelial cells is usually unaffected by mannose (mannose-resistant adherence) and is more common among strains exhibiting MRHA than among those exhibiting only mannose-sensitive hemagglutination (HA)[8] HA preparations are cytotoxic toward tissue culture cells.[9] The expected correlation between hemolysin and necrotoxin production is associated with the production of a "liquid" hemolysin, which is cytotoxic.[10] A close association has been observed between hemolysin production and cytotoxicity in 1 strain of *E. coli* (serotype 06, the standard hemolytic strain). The absence of strains that produce a "liquid" but not a "solid" hemolysin in UTIs indicates the importance of the production of non-diffusible hemolysins.[10] Recent studies have shown that HA and erythrocyte hemolysis can help bacteria to adhere to host cells and colonize them.[11] Cell surface hydrophobicity is one of the unspecific factors for bacteria to adhere to cell surfaces.[12] It is an important factor that helps *E. coli* adhere to various surfaces for colonization. Bacteria are lysed by normal serum due to the activity of the complement system. The alternate pathway of complement activation is potentially more important than the classical pathway. Bacterial resistance to killing by serum results from individual or combined effects of capsular polysaccharides, lipopolysaccharides and surface proteins.[13] Genes encoding urovirulence factors usually form gene clusters (also called as pathogenicity islands).[14]

Prevalence of virulent genes in UPEC vary according to the phylogenetic group, clinical conditions of host and geographical localization.[2] Given this generic virulence "arsenal" of UPEC, strains from different geographical regions show different disease severity and should be genetically different. Data on the genetic characteristics of UPEC are limited and UPEC are studied less in the west of Iran. Therefore, the present study was performed to determine some of the most important genes that control virulence factors in UPEC (*afa*, *pap* and *sfa* genes), with hemolysin, HA and hydrophobicity as the important phenotypic factors.

Materials and Methods

Study population and strains

E. coli strains were isolated from urine samples of 71 outpatients and 134 inpatients referred to different hospitals in west Iran (Kermanshah city). Clinical presentation of patients having lower urinary tract syndrome, including chills, fever, dysuria, frequency and sometimes urgency and cost vertebral angle tenderness, were noted. Urine samples were collected from clean-catch midstream urine. *E. coli* isolates were then identified using routine biochemical reactions. Suspicious isolates were confirmed using API20E kit.[15]

Deoxyribonucleic acid extraction and polymerase chain reaction assay

E. coli isolates were grown on Luria Bertani (LB) broth at 37°C overnight. DNA extraction was performed using the boiling method. To evaluate the purity of the extracted DNA, a spectrophotometer in ratio OD of 260/280 ≥ 1.7 was used. The quality of extracted DNA was controlled on 2% agarose gel. Amplification were performed in a thermal cycle C1000 (BioRad, Hercules, USA) under the following conditions. Multiplex PCR assays were performed in a final volume of 25 μL, containing DNA (5 μL boiling lysate), 1.5 mM MgCl2, 200 μM dNTP, 1.2 U Taq DNA polymerase (Fermentas), ×10 Taq DNA polymerase buffer and 0.45 μM primers. The reaction cycle included heating to 94°C for 10 min; 5 cycles of denaturation (94°C for 1 min), annealing (56°C for 1 min) and extension cycle (72°C for 4 min); and 25 cycles of denaturation (94°C for 40 s), annealing (61°C for 40 s) and extension (72°C for 3 min), followed by final extension (72°C for 5 min).[16,17] Expected lengths of the amplicons were ascertained by electrophoresis in 1% agarose gel. Bands were visualized in a GelDoc system (BioRad) under ultra violet light after ethidium bromide staining. Random samples of positive reaction were delivered to Fazabiotech Company for sequencing. Sequence analysis of PCR products was performed using a basic local alignment search tool search on the Genbank database [Table 1].

Hemolysin

Hemolysin test was performed according to the method described by Santo *et al*. In brief, bacteria were first cultured on LB broth and incubated for 16 h at 37°C. Next, a volume of 50 μl of bacterial suspension was inoculated in sheep blood agar and reincubated for 16 h at 37°C. Hemolysis production was verified by formation of a transparent halo around the spot.[18]

Surface hydrophobicity

In brief for this test, 50 µl of bacterial suspension was mixed with dilutions of 0.25, 0.5, 1, 1.4 and 2 M of ammonium sulfate. Isolates that formed colonies in 1.4 M or lower dilutions were considered as hydrophobic isolates. Sterile 96-well microplates were also used in this text.[19]

HA

Direct bacterial HA was performed for this test. In brief, bacteria were cultured on 1% nutrient broth and incubated at 37°C for 3-5 days to obtain the maximum production of fimbriae.[20]

Statistical analysis

Data entered in the Microsoft Access XP software were exported into the SPSS statistical software (version 16.0, SPSS, Inc., Chicago, IL), which was used for data analyzes. Categorical data were compared using a Chi-squared test. All *P* values were 2-sided, with a $P < 0.05$ considered to be statistically significant.

RESULTS

In total, 205 strains of *E. coli* were isolated from 42 (20.4%) male and 163 (79.5%) female with UTI. The age of patients ranged from 1 month to 107 years old (mean age, 31.88 ± 22.69 years) with the mean age for male 29.7 ± 40.9 and for female 19.9 ± 29.5. Of all the patients, 71 (34.6%) were out-patients and 134 (65.3%) were in-patients. Patients aged more than 40 years had more frequencies in both hospitals.

Virulence genotypes

Virulence factors such as *pap*, *afa* and *sfa* were analyzed by the PCR assay. Products of the PCR assay are shown in Figure 1. Frequencies of *pap*, *afa* and *sfa* were 42 (20.5%), 17 (8.3%) and 44 (21.5%), respectively. The results showed that 110 (53.7%)
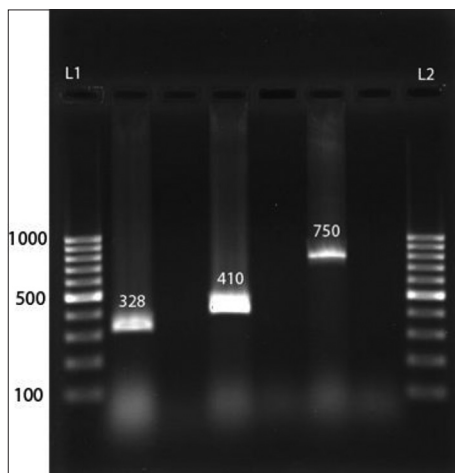

Figure 1: Polymerase chain reaction products shown on agarose gel. L1, deoxyribonucleic acid (DNA) size marker 100-bp; lane 2, pyelonephritis associated pili (328 bp), lane 4, S fimbrial adhesion (410 bp), lane 6, A fimbrial adhesion (750 bp); L2, DNA size marker 100-bp; and lanes 3, 5, 7, control negatives

isolates carried just 1 virulence factor, with 23 (11.2%) carrying *pap*, 10 (4.9%) carrying *afa* and 29 (14.1%) carrying *sfa*. 1 (0.5%) isolate carried all the 3 genes. However, no virulence factor was found in 123 (60%) isolates. There was a significant correlation between *pap* and the age of patients; the prevalence of *pap* was more common in patients aged 1-10 years ($P < 0.048$) [Table 2].

The result also showed correlation between the sex of patients and the *pap* gene, with the gene being more prevalent in male patients ($P < 0.051$) [Table 3].

Virulence phenotyping

Frequencies of HA, hemolysin and hydrophobicity were 138 (67.3%), 56 (27.3%) and 39 (19%), respectively. Among isolates showing HA, 103 (74.6%) were mannose resistant. 98 (47.8%) isolates were positive for HA, 8 (3.9%) for hemolysin and 4 (2%) for hydrophobicity. Our data indicated that there was a significant correlation between hemolysin and outpatients ($P < 0.024$) [Table 4] and between HA and

Table 1: Primer sequence and predicted length of amplified PCR products

Primer	Sequence (5′-3′)	Length of amplicons (bp)	Reference
pap	GAC GGC TGT ACT GCA GGG TGT GGC G ATA TCC TTT CTG CAG GGA TGC AAT A	328	[11,12]
sfa	CGG AGG AGT AAT TAC AAA CCT GGC A CTC CGG AGA ACT GGG TGC ATC TTA	410	[11,12]
afa	GCT GGG CAG CAA ACT GAT AAC TGT C CAT CAA GCT GTT TGT TCG TCC GCC G	750	[11,12]

PCR: Polymerase chain reaction

Table 2: Prevalence of virulence genes according to age groups

Virulence genes (%)	Age group (years)				
	1-10	11-20	21-30	31-40	>40
pap	11 (31.4)	2 (5.7)	11 (30.6)	7 (17.9)	11 (18.3)
sfa	4 (11.4)	9 (25.7)	8 (22.2)	12 (30.8)	11 (18.3)
afa	6 (17.1)	1 (2.9)	3 (8.3)	2 (5.1)	5 (8.3)
Hemaglutination	29 (82.9)	18 (51.4)	28 (77.8)	24 (61.5)	39 (65)
Mannose resistant hemaglutination	22 (62.9)	14 (40)	19 (52.8)	20 (52.8)	28 (46.7)
Hemolysin	8 (22.9)	12 (34.3)	9 (25)	13 (33.3)	14 (23.3)
Hydrophobicity	5 (14.3)	9 (25.7)	7 (19.4)	4 (10.3)	14 (23.3)

Table 3: Prevalence of virulence genes and their relation with sex and source of bacteria

Virulence genes (%)	Sex			Hospital		
	Male	Female	P	Outpatient	Inpatient	P
pap positive	13 (31)	29 (17.8)	0.051	29 (21.6)	13 (18.3)	0.356
sfa positive	6 (14.3)	38 (23.3)	0.144	29 (21.6)	15 (21.1)	0.541
afa positive	4 (9.5)	13 (8)	0.474	11 (8.2)	6 (8.5)	0.573

Table 4: Prevalence of virulence factors according to sex and hospital

Virulence genes (%)	Sex			Hospital		
	Male	Female	P	Outpatient	Inpatient	P
Hemaglutination positive	33 (78.6)	105 (64.4)	0.057	93 (69.4)	45 (63.4)	0.236
Mannose resistant hemaglutination positive	28 (66.7)	75 (46)	0.058	70 (52.2)	33 (46.5)	0.663
Non-hemaglutination	9 (21.4)	58 (35.6)		41 (30.6)	26 (36.6)	
Hemolysin positive	11 (26.2)	45 (27.6)	0.511	43 (32.1)	13 (18.3)	0.024
Hydrophobicity positive	7 (16.7)	32 (19.6)	0.425	33 (24.6)	6 (8.5)	0.003

the age of patients; it was more common in patients aged 1-10 years and minimum in patients aged 10-20. Frequencies of isolates with genes *pap*, *afa* and *sfa* and phenotypic factors such as HA, hemolysin and hydrophobicity were 1 (0.5%) and 30 (14.6%) isolates carried none of the genes or phenotypic factors [Table 2]. Statistical significance of co-occurrence of 2 virulence factors was assessed using χ^2 and Fisher's exact test. A significant difference was found for *pap-sfa* ($P < 0.033$), *pap-hly* ($P < 0.028$), *sfa-hly* ($P < 0.000$), *sfa-hydro* ($P < 0.005$), *hly-hydro* ($P < 0.000$) and *hemo-MRH* ($P < 0.001$); co-occurrence was tested for the same sample [Table 5].

Discussion

UPEC are a major cause of UTIs and may be responsible for approximately 90% of UTIs. In the present study, we found that 20.5%, 8.3% and 21.5% *E. coli* isolates from urine samples of in- and out-patients with UTI were positive for the *pap*, *afa* and *sfa* genes, respectively. Frequencies of isolates with HA, hemolysin and hydrophobicity were 67.3%, 27.3% and 19%, respectively. Various studies investigated the prevalence of virulence factors in *E. coli* isolates from patients with UTI. A study from Ankara, similar to our study, showed that almost 23% and 9.94% *E. coli* isolates were positive for *pap* and *afa* genes, respectively. However, there was a significant difference between the frequencies of *sfa* gene (6.21% versus 21.5%)[21] In both studies, a significant correlation was observed between *pap* and *sfa* genes. Bogylova in Slovaki, in the same investigation, reported that 8.3% extraintestinal *E. coli* isolates were positive for the *afa* gene, which is exactly same as our result. However, there is a significant difference between 2 other gene frequencies (74% and 65% for *pap* and *sfa* genes, respectively).[22] These differences may be due to the differences in geographical regions. In Iran (Shiraz), Farshad reported 30.2% and 18.75% frequencies for *pap* and *sfa* genes, respectively. In another study in Shiraz, Emamghoreishi reported a frequency of 14.6% for the *sfa* gene.[23] In our study, a significant difference was detected between age groups infected with *pap*-positive bacteria ($P < 0.043$). Patients aged 1-10 and 21-30 years were more infected with *pap*-positive *E. coli* (31.4% and 30.6%, respectively) [Table 2]. The *pap* gene encodes for P-fimbriae that allows bacteria to adhere to epithelial surfaces and protect them against urine lavage,

Table 5: significant relations between six virulence factors

Virulence factors	Negative n (%)	Positive n (%)	P
pap			
Sfa−	133 (81.6)	28 (66.7)	0.033
sfa+	30 (18.4)	14 (33.3)	
afa−	152 (93.3)	36 (85.7)	NS
afa+	11 (6.7)	6 (14.3)	
hly−	124 (76.1)	25 (59.5)	0.028
hly+	39 (23.9)	17 (40.5)	
hydro−	135 (82.8)	31 (73.8)	NS
hydro+	28 (17.2)	11 (26.2)	
haema−	57 (35)	10 (23.8)	NS
haem+	106 (65)	32 (76.2)	
MRH	79 (48.5)	24 (57.1)	NS
MSH	27 (16.6)	8 (19)	
Sfa			
afa−	146 (90.7)	42 (95.5)	NS
afa+	15 (9.3)	2 (4.5)	
hly−	130 (80.7)	19 (43.2)	0.000
hly+	31 (19.3)	25 (56.8)	
hydro−	137 (85.1)	29 (65.9)	0.005
hydro+	24 (14.9)	15 (34.1)	
haema−	54 (33.5)	13 (29.5)	NS
haem+	107 (66.5)	31 (70.5)	
MRH	78 (48.4)	25 (56.8)	NS
MSH	29 (18)	6 (13.6)	
Afa			
hly−	135 (71.8)	14 (82.4)	NS
hly+	53 (28.2)	3 (17.6)	
hydro−	152 (80.9)	14 (52.4)	NS
hydro+	36 (19.1)	3 (17.6)	
haema−	60 (31.9)	7 (41.2)	NS
haem+	128 (68.1)	10 (58.8)	
MRH	98 (52.1)	5 (29.4)	NS
MSH	30 (16)	5 (29.4)	
hly			
hydro−	140 (94)	26 (46.4)	0.000
hydro+	9 (6)	30 (53.6)	
haema−	46 (30.9)	21 (37.5)	
haem+	103 (63.1)	35 (62.5)	
MRH	75 (50.3)	28 (50)	NS
MSH	28 (18.8)	7 (12.5)	
hydro			
haem−	50 (30.1)	17 (43.6)	NS
haem+	116 (69.9)	22 (56.4)	
MRH	87 (52.4)	16 (41)	NS
MSH	29 (17.5)	6 (15.4)	
haema			
MRH	0 (0)	103 (74.6)	0.001

NS: Non-significant

thus allowing them to ascend further to even cause a serious disease. In the first decade of life, hygiene problems are the most important reason for bacterial infection, particularly in girls because of a short urethra (approximately 4 cm). The third decade of life is a time of sexual activity.

Previous studies have shown that UPEC are transmitted during sexual activity.[16,17] Our results highlight higher frequency of HA (67.3%) than other virulence factors, indicating a crucial role of virulence factors in UTI-causing *E. coli*. Therefore, it appears that virulence factors are the most important armament to adhere to epithelial surface and to break host defense system. The ability of bacteria to adhere to urinary epithelium can protect them from urinary lavage. HA is significantly correlated with MRH ($P < 0.001$). Prevalence of isolates that have HA was 67.3% either alone (47.8%) or in association with 2 other phenotypic factors (11.2%).

In total, 3.9% isolates were only positive for hemolysin and 2% were positive for hydrophobicity. Only 1 (0.5%) of the UPEC isolates was positive for all 6 virulence factors. These observations confirm the higher prevalence of HA among our isolates and probably the more significant role in pathogenicity in this geographical region. In Raksha's study on phenotypic virulence factors in *E. coli* in India, with prevalence of HA was 30.9% and hemolysin with 41.36%, have had the most frequency among phenotypic pathogenic factors.[24] Although HA is the most important factor in our isolates, it does not cause a serious disease leading to hospital admission. We found a significant difference between in- and out-patients infected with HA-positive isolates (18.3% in-patients versus 32.1% outpatients; $P < 0.024$). Therefore, it appears that in our area, HA has a significantly higher prevalence and perhaps plays a crucial role in the pathogenicity of our isolates.

Due to differences in social, economic, health, hygiene and environmental conditions between developed and developing countries, more studies are needed from developing countries to detect the virulence factors of UPEC to determine the pattern of pathogenicity in these bacteria. Furthermore, studies are needed to determine the relationship between the expression of virulence factors and antibiotic resistance. These studies will be important in understanding the role of these factors in causing UTIs, which in turn may lead to the development of universal vaccines to prevent such infections.

Acknowledgments

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