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Prevalence of virulence genes in *Escherichia coli* strains isolated from Romanian adult urinary tract infection cases

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Abstract

A total of 78 *E. coli* strains isolated from adults with different types of urinary tract infections were screened by polymerase chain reaction for prevalence of genetic regions coding for virulence factors. The targeted genetic determinants were those coding for type 1 fimbriae (*fimH*), pili associated with pyelonephritis (*pap*), S and F1C fimbriae (*sfa* and *foc*), afimbrial adhesins (*afa*), hemolysin (*hly*), cytotoxic necrotizing factor (*cnf*), aerobactin (*aer*). Among the studied strains, the prevalence of genes coding for fimbrial adhesive systems was 86 %, 36%, and 23% for *fimH*, *pap*, and *sfa/foc,*respectively. The operons coding for *Afa* afimbrial adhesins were identified in 14% of strains. The *hly* and *cnf* genes coding for toxins were amplified in 23% and 13% of strains, respectively. A prevalence of 54% was found for the *aer* gene. The various combinations of detected genes were designated as virulence patterns. The strains isolated from the hospitalized patients displayed a greater number of virulence genes and a diversity of gene associations compared to the strains isolated from the ambulatory subjects. A rapid assessment of the bacterial pathogenicity characteristics may contribute to a better medical approach of the patients with urinary tract infections.

Keywords: *Escherichia coli* - virulence factors - PCR - urinary tract infections

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Introduction

Urinary tract infection (UTI) having as etiologic agent pathogenic *Escherichia coli* remains a common and troublesome health problem in many different countries all around the world, resulting in considerable morbidity and expense. Its propensity to recur and the serious challenge posed to effective chemotherapy make it worth studying the virulence repertoire of *E. coli* strains isolated from UTIs. Well recognized virulence factors, such as fimbrial and afimbrial adhesins, toxins and siderophores and new potential ones provide means for *E. coli* strains to disrupt the host health equilibrium [1].

The advances in molecular technology have facilitated apprehensive studies regarding uropathogenic *E. coli* [2-4]. The rapid assessment of virulence determinants detected by polymerase chain reaction (PCR) may be useful for diagnosis and therapeutic strategies [5-7]. The aims of this study were: (i) to evaluate the prevalence of different operons coding for virulence factors among *E. coli* strains isolated from the urine of adults with UTIs, (ii) to establish a data bank containing the molecular characteristics of *E. coli* strains circulating in Romania, and (iii) to attempt a correlation between the genetic virulence traits of the clinical isolates and the type of urinary tract infection. The results may contribute to improving the management of UTI.

The genetic determinants studied were those coding for type 1 fimbriae (*fimH*), pili associated with pyelonephritis (*pap*), S and F1C fimbriae (*sfa* and *foc*), afimbrial adhesins (*afa*), hemolysin (*hly*), cytotoxic necrotizing factor (*cnf*), and aerobactin (*aer*).

Materials and methods

Bacterial strains and subjects

We studied a total of 78 strains of *E. coli* isolated from adults between November 1999 and April 2000 (62 women aged 18 to 84 years and 16 men aged 31 to 86 years). Fifty-two of the subjects were hospitalized (Clinical Hospital of Nephrology " Dr. C. Davila", Bucharest and Urology Department of Clinical Institute "Prof. Dr. Th. Burghele", Bucharest) and 26 were ambulatory patients. The diagnosis of UTI was established, based on clinical symptoms and laboratory investigations. The laboratory criterion for acute *E. coli* UTI was defined as the presence of a positive urine culture with at least 10^5 CFU of *E. coli* per ml of clean-voided urine. Among the hospitalized patients there were 12 cases of asymptomatic bacteriuria (no UTI symptoms, urine culture $>10^5$ CFU/ml), 29 cystitis cases (dysuria, with or without suprapubic pain, with no fever), 2 cases of acute pyelonephritis (flank pain, fever, with or without chills, nausea, vomiting; laboratory findings: significant leukocytosis, nonspecific acute phase reactants, such as elevated C-reactive protein, proteinuria, pyuria with leukocyte casts in urine) and 9 cases of chronic pyelonephritis (suggestive impairment in renal concentrating ability and ultrasound/pyelography evidence of asymmetric kidneys, pyelo-caliceal distorsion, and cortical scarring). All the ambulatory subjects presented symptomatic lower tract infections.

The isolation and identification of *E.coli* strains was performed by minimal standard bacteriological tests (in the clinical laboratories of the two hospitals and in the clinical laboratory of the Public Health Centre). It was confirmed in the *E. coli* Reference Laboratory of Cantacuzino Institute using conventional biochemical tests [8].

Strain A30 [9] was used as positive control for *afa* operon and the strain J96 [10] was the positive control for *pap*, *sfa/foc*, *hly*, *cnf* and *fimH* sequences. The positive control for *aer* PCR was HB101(pABN1) [11]. *E. coli* strain HB101 [12] was the negative control for all the DNA amplifications, except for *fimH* amplification, where we used the strain AAEC185[13].

Bacterial cultures and DNA extraction

E. coli strains were grown in Luria-Bertani (LB) broth at 37°C overnight. Bacteria were pelleted from 1.5 ml LB broth, suspended in 200 µl sterile distilled water and boiled at 100°C for 15 min. Following centrifugation of the lysate, a 150 µl sample of the supernatant was stored at - 20°C as a template DNA stock.

PCR

Specific primers were used to amplify sequences of the *fim*, *pap*, *sfa/foc*, *afa*, *hly*, *cnf*, and *aer* operons. Details of primer sequences, predicted sizes of the amplified products and specific annealing temperatures are given in Table 1. Detection of *pap*, *sfa/foc* and *afa* sequences was done by multiplex PCR [7].

PCR amplification of bacterial DNA extracts was done in a total volume of 50 µl containing 10 µl template DNA, 30 pmoles of each of the primers, the four deoxynucleoside

Table 1 Primers for PCR assays.

Virulence factor	Target gene(s)	Name	Primer sequence (5'-3')	Size of amplicon (bp)	Annealing temperature (°C)	Reference
Type 1 fimbriae	fimH	fimH-f fimH-r	AACAgCgATgATTTCCAgTTTgTgTg ATTgCgTACCAgCATTAgCAATgTCC	465	60	James R. Johnson[a]
P fimbriae	papC	pap1 pap2	gACggCTgTACTgCAgggTgTggCg ATATCCTTTCTgCAgggATgCAATA	328	65	7
S and F1C fimbriae	sfa/focDE[b] region	sfa1 sfa2	CTCCggAgAACTgggTgCATCTTAC CggAggAgTAATTACA AACCTggCA	410	65	7
Afa adhesins	AfaC[c]	afa-f afa-r	CggCTTTTCTgCTgAACTggCAggC CCgTCAgCCCCCACggCAgACC	672	65	9
Haemolysin	hlyCA region	hly s hly as	AgATTCTTgggCATgTATCCT TTgCTTTgCAgACTgTAgTgT	556	65	29[d]
Cytotoxic necrotizing factor	cnf	cnf s cnf as	TTAT ATAgTCgTCAAgATggA CACTAAgCTTTACAATATTgA	693	50	Eric Oswald[a]
Aerobactin	iucC	aer s aer as	AAACCTggCTTACgCAACTgT ACCCgTCTgCAAATCATggAT	269	55	29

[a] Personal communication.
[b] The same primers are used to detect the *sfa* and *foc* operons, which are highly related (7)
[c] The same primers also detected the *dra* operon (encoding the Dr adhesin) highly related to the *afa* operons (9).
[d] Modified according Genbank accession no X02768

triphosphates (each at 200 µM) and 1.25 U Taq DNA polymerase (Amersham USB) in 1x PCR buffer containing 1.5 mM $MgCl_2$. The amplification procedure consisted in an initial denaturation at 94°C for 5 min in a Mastercycler Eppendorf 5330, followed by 25 cycles of denaturation at 94°C for 30 sec, annealing at specific temperature for 30 sec, and extension at 72°C for 1 min with a 2 sec increase for sequential cycles. Aliquots (10µl) of the final reaction mixture underwent gel electrophoresis in 2% agarose containing 0.5 µg of ethidium bromide per ml. Amplified DNA fragments of specific sizes were detected by UV induced fluorescence. The sizes of the amplicons were estimated by comparing them with the 1 kb DNA ladder (Gibco BRL) which was run on the same gel.

Results

The prevalence of virulence genes ranged from 13% for *cnf* to 86 % for *fimH* (Table 2). Of the adhesin coding genes, *fimH* was the most prevalently detected (67 strains), followed by *pap* (28 strains) and *sfa/foc* (18 strains), respectively. Of the toxin coding genes studied, *hly* (18 strains) was more prevalent than *cnf* (10 strains). The *aer* operon coding for the aerobactin siderophore was broadly distributed, being the second detected virulence factor coding gene after the highly prevalent *fimH* gene. Depending on the number of *E. coli* strains screened for each of the two groups of subjects (hospitalized and ambulatory subjects) the prevalence of the targeted genes was similar for the *aer* and *fim* operons. The *pap* operon was detected more frequently in the strains isolated from the ambulatory subjects. As for the *sfa/foc*, *hly* and *cnf* sequences, the percentage of PCR positive strains was greater in the strains from hospitalized subjects. The *afa* sequences were not amplified in any of the strains from the ambulatory collection.

Based on the distribution of the various targeted sequences all the studied strains exhibited 21

Table 2 Prevalence of virulence factor associated genes among 78 *E. coli* isolates from patients with UTI.

Operon or gene	No. of positive strains in patients		Total no. (%) of positive strains
	hospitalized	ambulatory	
fimH	45	22	67 (86)
aer	27	15	42 (54)
pap	17	11	28 (36)
sfa/foc	15	3	18 (23)
hly	14	4	18 (23)
afa	11	-	11 (14)
cnf	9	1	10 (13)

virulence gene patterns, referred to as Ec followed by an Arabic numeral (Table3). The strains in which no virulence gene was detected were included in the Ec1 pattern. Two of the virulence gene patterns designated as Ec2 and Ec3 were characterized by the presence of only one gene, which was either *fimH* (19 strains) or *aer* (5 strains). Five patterns (Ec4 - Ec8) were represented by strains possessing a two gene association (15 strains). The patterns which included strains presenting three virulence genes (Ec9 - Ec12) were the best represented (19 strains). The association of four genes was recognized in Ec13 - Ec16 patterns (4 strains), followed by the Ec17 -Ec20 patterns, which encompassed the five gene positive strains (10 strains). Among all the studied strains, three strains exhibited Ec21 pattern, characterized by the association of six genes.

Table 3 Virulence patterns identified among the studied strains.

Pattern	Virulence operon or gene							No. of strains
	fim	*pap*	*sfa/foc*	*afa*	*hly*	*cnf*	*aer*	
Ec1	-	-	-	-	-	-	-	3
Ec2	+	-	-	-	-	-	-	19
Ec3	-	-	-	-	-	-	+	5
Ec4	+	-	-	-	-	-	+	8
Ec5	+	-	-	+	-	-	-	3
Ec6	+	+	-	-	-	-	-	2
Ec7	+	-	+	-	-	-	-	1
Ec8	+	-	-	-	+	-	-	1
Ec9	+	-	-	+	-	-	+	7
Ec10	+	+	-	-	-	-	+	10
Ec11	-	+	+	-	-	-	+	1
Ec12	+	-	-	-	+	-	+	1
Ec13	+	+	+	-	+	-	-	1
Ec14	-	+	+	-	+	-	+	1
Ec15	+	+	-	+	-	-	+	1
Ec16	+	-	+	-	+	+	-	1
Ec17	+	+	+	-	+	+	-	4
Ec18	+	+	+	-	+	-	+	4
Ec19	-	+	+	-	+	+	+	1
Ec20	+	-	+	-	+	+	+	1
Ec21	+	+	+	-	+	+	+	3

Table 4 Distribution of virulence gene patterns in relationship with different types of UTI.

UTI type	No. of detected genes	Pattern	No. of strains	
			Hospitalized subjects	Ambulatory subjects
Asymptomatic bacteriuria	0	Ec1	1	
	1	Ec2	4	
	2	Ec4	3	
		Ec7	1	
	3	Ec9	1	
	4	Ec14	1	
	5	Ec20	1	
Cystitis	0	Ec1	-	2
	1	Ec2	5	7
		Ec3	2	2
	2	Ec4	1	3
		Ec5	2	
		Ec6	1	
		Ec8	1	
	3	Ec10	2	8
		Ec9	4	
		Ec12	-	1
	4	Ec13	1	
		Ec15	1	
		Ec16	1	
	5	Ec17	2	1
		Ec18	2	2
		Ec19	1	
	6	Ec21	3	
Acute pyelonephritis	1	Ec2	1	
	5	Ec17	1	
Chronic pyelonephritis	1	Ec2	2	
		Ec3	1	
	2	Ec4	1	
		Ec5	1	
		Ec6	1	
	3	Ec9	2	
		Ec11	1	

Table 4 presents the distribution of virulence factor association patterns in relationship with the different types of UTI. One of the 12 strains isolated from asymptomatic bacteriuria exhibited Ec1 pattern. The other 11 strains carried between one to five genes variously associated in six patterns. Four strains displayed only *fimH* (Ec2 pattern). In other three strains *fimH* was associated with *aer* (Ec4 pattern), and each of the rest of positive strains were represented by a distinct pattern (Ec7, Ec9, Ec14, Ec20).

Among the strains isolated from patients with cystitis the multitude and diversity of genes was the greatest. Seventeen of the 21 genetic virulence patterns were identified in this collection. The Ec2 and Ec10 patterns were the most frequently detected, being identified in 12 and 10 strains, respectively.

The two strains isolated from acute pyelonephritis expressed the Ec2 and Ec17 pattern, respectively.

The strains isolated from chronic pyelonephritis presented one, two or three virulence genes. The genes coding for toxins were not detected among these strains. Two strains were identified as Ec2 pattern and other two strains showed the Ec9 pattern. The remaining gene combinations detected in these strains were equally distributed between Ec3, Ec4, Ec5, Ec6 and Ec11 patterns, respectively.

Discussion

The urinary tract infections which are not properly managed from their onset can become in time a real threat, finally leading to renal failure. This is partly due to the fact that urinary signs and symptoms are often not useful to reliably distinguish upper and lower UTIs [14]. A better knowledge of virulence characteristics of the microorganism causing the infection allows the clinician to anticipate up to a point the evolution of infection in the host organism.

Fimbriae-mediated adherence, involving type 1, P, S, and F1C fimbriae is important for the virulence of *E. coli* in the urinary tract [15-17]. Among the Romanian strains the distribution of the genes encoding these various types of fimbriae was in agreement with the published data from other reports [18]. Consistent with these data type 1 fimbriae operon was, as expected, the most prevalent virulence factor identified. The *fimH* negativity by PCR method did not point to the absence of *fim* operon in the strains. An argument for its presence was the identification of *fimA* gene in four of the eleven *fimH* negative strains (data not shown). The well-characterized variability of FimH adhesin gene could account for our inability to detect it [19]. The distribution of the F1C or S fimbriae-encoding operons found among the studied strains was also similar with previously reported data [18]. Regarding the P fimbriae, pooled results from many studies indicate that among *E. coli* strains from patients with acute pyelonephritis and cystitis, who lack underlying medical risk of infection, approximately 80% and 30%, respectively, possess P fimbriae [1,18]. Although the frequency of *papC* in cystitis strains is in accordance with literature data, we found that only 27% of the pyelonephritis strains contain *pap* sequences. It is noteworthy that most of the pyelonephritis strains studied were isolated from patients with chronic infections. Additional epidemiological studies need to be carried out to confirm this observation.

A growing importance is attributed to the *Afa* afimbrial adhesins, which have been implicated in the development of chronic interstitial nephritis [20]. We found a higher percentage of *afa* PCR-positive strains among isolates from patients with chronic pyelonephritis (33.3%) than among strains associated with cystitis (12.7%) or asymptomatic bacteriuria (8.3%).

The prevalence of *hly* and *cnf* operons encoding two toxins implicated in tissue damage and dysfunction of local immune responses [21, 22] and the prevalence of aerobactin operon *aer*, which confers the ability to acquire iron [23], among our collection of clinical isolates fit with those found by other investigators [18].

The strains isolated from patients admitted in hospital exhibited a greater number of virulence coding genes per strain as well as a great diversity of gene patterns and seemed to be more aggresive than strains from ambulatory patients. This observation may point to the concordance of the severity of the symptoms with the number of virulence genes. The maximum number of detected amplicons in one strain was six out of seven virulence gene regions targeted. There was no presence of *sfa/foc* gene and *afa* in a same strain. Instead, the presence of *afa* together with *aer* gene was commonly detected in the same strain, as in Ec9 and Ec15 patterns, an association previously signaled [24]. A co-dependence of these virulence factors in a particular pathogenic pathway, which may differ from that used by the *E. coli* strains expressing fimbrial adhesins and toxins was suspected [24, 25]. Some of the virulence patterns found in the studied strains could be suggestive for the presence of pathogenicity islands described in uropathogenic *E. coli* [26-28]. We amplified in the same strain *pap*, *sfa/foc* and *hly* or *pap*, *sfa/foc*, *hly* and *cnf*, as presented in Table 3. However, this observation needs further sequencing research to determine if the pathogenic determinants are linked on the bacterial chromosome.

This first molecular study of *E. coli* strains isolated from UTI was meant as a step towards improving the knowledge regarding their virulence genetic determinants. The molecular features of *E. coli* extraintestinal strains revealed by its results may contribute to a better medical approach of the patients concerned.

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