Research article/Raziskovalni prispevek

PREVALENCE OF TOXIN ENCODING GENES IN *ESCHERICHIA COLI* ISOLATES FROM URINARY TRACT INFECTIONS IN SLOVENIA

PREVALENCA GENSKIH ZAPISOV ZA TOKSINE V IZOLATIH BAKTERIJE *ESCHERICHIA COLI,* PRIDOBLJENIH IZ VZORCEV URINA V SLOVENIJI

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Abstract

Methods *110 uropathogenic Escherichia coli strains (UPEC) obtained from the Institute of Microbiology and Immunology of the Medical Faculty in Ljubljana were screened by PCR with primers specific for the following toxin encoding genes: hlyA (haemolysin), cnf1 (cytotoxic necrotising factor 1), usp (uropathogenic specific protein USP) and ibeA (invasin). Dot blot hybridisation experiments were performed to validate the PCR assays.*

Results *In 44% of the strains usp gene sequences were detected. The prevalence of hlyA and cnf1 was 25% and 23%, respectively. Only 9% of the strains harbored ibeA. The majority of the tested toxin encoding genes was found in strains belonging to the B2 phylogenetic group.*

Conclusions *The toxin encoding genes hlyA, cnf1 and usp were strongly co-associated. Further, we found a statistically significant co-association of ibeA and usp. The prevalence of the tested toxin encoding genes in E. coli strains from urinary tract infections isolated in Slovenia is comparable to those from studies in other geographic regions.*

Key words *uropathogenic Escherichia coli; UPEC; toxins; hlyA; cnf1; ibeA; usp genes*

Izvleček

Izhodišča *Bakterija Escherichia coli (E. coli) je enterobakterija, ki živi v spodnjem delu prebavila človeka in toplokrvnih živali. Kljub temu da je E. coli del normalne mikrobne flore, lahko povzroči okužbe. Pestrost obolenj, v katere je vpletena E. coli, je precejšnja, saj lahko povzroča drisko, vnetje sečnika in ledvic, pljučnico, vnetje možganskih ovojnic, okužbe ran in drugo. Patogeni sevi se razlikujejo od nepatogenih po tem, da imajo v svojem genomu genske zapise za virulentne dejavnike (toksine, adhezine, kapsule ...). Okužba sečil je ena izmed najpogostejših bakterijskih infekcij in E. coli povzroča večino teh okužb. Zaradi pogostnosti pojavljanja okužb v sečilih so virulentni dejavniki sevov E. coli, ki povzročajo te okužbe (UPEC – uropatogena E. coli), za preučevanje zelo zanimivi. Kar nekaj toksinov: alfa-hemolizin (HlyA), citotoksični nekrotizirajoči dejavnik (CNF1), invazin (IbeA) in uropatogeni specifični protein (USP), povezujejo s patogenostjo sevov UPEC. Zbrane informacije o pogostnosti zapisov za toksine bi lahko bile tudi osnova za diagnostiko okužb z UPEC sevi.*

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Metode *Proučevali smo 110 uropatogenih sevov bakterije E. coli, ki smo jih na Inštitutu za mikrobiologijo in imunologijo Medicinske fakultete v Ljubljani osamili iz diagnostičnih vzorcev urina, z namenom določiti gene, ki imajo zapise za nekatere značilne bakterijske toksine: hlyA (hemolizin), cnf1 (citotoksični nekrotizirajoči faktor 1), ibeA (invazin) in usp (uropatogeni specifični protein USP). Uporabili smo metodo verižne reakcije s polimerazo (PCR) z začetnimi oligonukleotidi, specifičnimi za posamezne proučevane genske zapise za toksine. Zanesljivost uporabljene metode PCR smo preverili z metodo hibridizacije točkovnega odtisa.*

Rezultati *Pri 44 % sevov smo dokazali gen usp, prevalenca gena hlyA je bila 25 % in gena cnf1 23 %. Samo pri 9 % sevov smo dokazali gen ibeA za invazin. Večino najdenih genskih zapisov za toksine smo našli v sevih filogenetske skupine B2. Medsebojna povezanost pojavljanja genskih zapisov za toksine HlyA, CNF1 in USP je bila statistično značilna. Pojavljanje genskega zapisa za ibeA je bil statistično značilno povezano samo s pojavljanjem genskega zapisa za usp.*

Zaključki *Dobljeni rezultati kažejo, da so proučevani genski zapisi za toksine pri preiskovanih UPEC sevih prisotni v podobni meri kot pri podobnih tujih raziskavah. Ker nobena izmed že objavljenih raziskav ni vključevala vseh štirih v tej raziskavi proučevanih genskih zapisov, je ta raziskava prva, ki je pokazala statistično značilno povezanost ibeA in usp.*

Ključne besede *uropatogena Escherichia coli; UPEC; toksini; geni hlyA; cnf1; ibeA; usp*

Introduction

Urinary tract infections (UTIs) are one of the most frequent bacterial infections especially in women. Up to one third of women will suffer from an episode of UTI during their lifetime.[1] UTI is diagnosed by either clinical observation or isolation of the causative microorganism from pathologic urine.[2] In Slovenia uropathogenic *Escherichia coli (E. coli)* strains (UPEC) cause approximately 80 % of uncomplicated urinary tract infections.[3] The ability of uropathogenic strains to cause diseases is due to possession of virulence factors: adhesins, toxins, polysaccharide coatings, invasins and iron uptake systems.[4] It is essential to link a pathogen's virulence factors to clinical manifestations, especially recurrent UTIs.

In pathogenic *E. coli* strains several important toxins have been identified. The best known are alpha hemolysin (HlyA) and cytotoxic necrotizing factor 1 (CNF1), which have been associated with UPEC strains.[5] Well known toxins are also invasins, the Ibe proteins that help *E. coli* strains to invade the human brain microvascular endothelial cells.[6] The presence of IbeA protein is statistically significantly higher in strains causing cystitis and/or pyelonephritis.[7] The gene for the uropathogenic specific protein (USP) that was found as a homologue of the *Vibrio cholerae* zonula occludens toxin encoding gene,[8] has been significantly more often detected in UPEC strains than in fecal strains from healthy individuals.[9]

To diminish the burden of UPEC, using effective preventive measures, data on virulence factor prevalence in different geographic regions must be assessed. Since bacterial toxins are a potentially good target for a vaccine a number of studies analysing the prevalence of virulence factors, including toxins, in different UPEC have been performed.[10–12] As no such data was available for the geographic region of Slovenia, the aim of the present study was to analyse the prevalence of toxin encoding genes in 110 *E. coli* strains causing UTI in Ljubljana, Slovenia.

Material and methods

Bacterial strains

A total of 110 *E. coli* isolates (designated DL 1 to DL 110) from diagnostic urine samples of people with urinary tract infections treated in 2002, at the Institute of Microbiology and Immunology, Medical Faculty, Ljubljana, were studied. Only one isolate from each patient was analysed.

Ninety-four (86 %) of the patients were women. The serotype, phylogenetic groups and traits typical of horizontal gene transfer (*traT,* integrons, *rep*) were determined previously.[13]

Detection of toxin encoding genes

The primers and PCR conditions used to amplify genes encoding toxins with polymerase chain reaction (PCR) are listed in Table 1. Lysates for PCR were prepared by boiling, according to Le Bouguenec et al.,[17] of overnight cultures grown in liquid Luria Bertani (LB) medium. Amplification was performed in an automated thermal cycler (UNOII, Biometra, Göttingen, Germany) in a 50 µl reaction mixture containing template DNA (10 µl of boiled lysate), 20 pmol of forward and reverse primer, 0.2 mM of dNTP mixture, 1.25 U *Taq* DNA polymerase and 2.5 mM $MgCl_2$ in 1× PCR buffer (Fermentas, Vilnius, Lithuania).

Dot blot hybridisation experiments using the »DIG DNA Labelling and Detection kit« (Roche, Mannheim, Germany) were performed to validate the PCR assays. Probes were prepared using the same primers as for the PCR experiments and labelled with digoxigenin. The template DNA samples were the same as in the PCR experiments.

Table 1. *Oligonucleotide primers and PCR conditions to detect toxin encoding genes.*
Razpr. 1. *Oligonukleotidni začetniki in pogoji PCR za ugotavljanje genskih zapisov za toksine.*

Gene Gen	Oligonucleotide sequence (5' to 3') Oligonukleotidno zaporedje (od 5' do 3')	Size of product (bp) Velikost produkta (bp)	PCR conditions Pogoji PCR			Reference Referenca
hlyA	aacaaggataagcactgttctggct accatataagcggtcattcccgtca	1177	95 °C	2.5 min	1×	14
			94 °C 64 °C 72 °C	0.5 min 0.5 min 1.5 min	30 ×	
			72 °C	7 min	1 ×	
cnf1	ctgacttgccgtggtttagtcgg tacactattgacatgctgcccgga	1295	94 °C	4 min	1 ×	15
			94 °C 59 °C 72 °C	1.5 min 1.5 min 2 min	30 ×	
			72 °C	5 min	1 ×	
ibeA	aggcaggtgtgcgccgcgtac tggtgctccggcaaaccatgc	170	94 °C	2.5 min	1×	11
			94 °C 63 °C 72 °C	0.5 min 0.5 min 3 min	25 ×	
			72 °C	10 min	1 ×	
usp	atgctactgtttccgggtagtgtgt catcatgtagtcggggcgtaacaat	1000, 2500, 3000	95 °C	2.5 min	1 ×	16
			94 °C 68 °C 72 °C	0.5 min 1 min 1 min	30 ×	
			72 °C	7 min	1 ×	

Statistical analysis

The significance of the results was established using the Fisher's exact test (2-tailed) available on-line on the web site http://www.matforsk.no/ola/fisher.htm and the level of significance was set at a P value < 0.05. To reveal the degree of association the Pearson's correlation coefficient was calculated on-line on the web site http://www.quantativeskills.com/sisa/statistics/twoby2.htm. Interpretation of the correlation coefficient was as follows: 0.1 to 0.29 – small correlation, 0.3 to 0.49 – medium correlation and 0.5 to 1 – large correlation.

Results

Prevalence of toxin encoding genes

The presence of toxin encoding genes in the genomes of DL strains was screened by PCR and validated in the hybridization experiments. Figure 1 gives an example for *ibeA* detection. Among the screened toxin encoding genes the *usp* gene had the highest prevalence as *usp* specific nucleotide sequences were detected in 48 strains (44 %). The prevalence of *hlyA* and *cnf1* was similar, 28 (25 %) and 25 strains (23 %), respectively, possessed the tested nucleotide sequences. Only 10 strains (9 %) harbored *ibeA* sequences (Figure 2).

Distribution of toxin encoding genes among phylogenetic groups

E. coli isolates can be divided into four main phylogenetic groups A, B1, B2 and D.[18] Analysis of the distribution of toxin encoding genes among the previously determined phylogenetic groups of studied strains[13] revealed that the tested toxin encoding genes *hlyA, cnf1, ibeA* and *usp* are mostly harbored by UPEC strains belonging to the B2 phylogenetic group (Table 2).

Table 2. *Distribution of toxin encoding genes among E. coli phylogenetic groups.*
The prevalence of toxin encoding genes among phylogenetic groups is given as the total number N and % of found toxin encoding gene sequences in different phylogenetic groups.

Razpr. 2. *Razporeditev genskih zapisov za toksine po filogenetskih skupinah E. coli.*
Prevalenca genskih zapisov za toksine v posameznih filogenetskih skupinah je podana kot število N in % najdenih genskih zapisov za toksine v različnih filogenetskih skupinah.

Toxin encoding gene Genski zapis za toksin	Prevalence N (%) in phylogenetic group Prevalenca N (%) v filogenetski skupini				Total Skupaj
	A (N = 28)	B1 (N = 6)	B2 (N = 55)	D (N = 21)	(N = 110)
hlyA	1 (4)	0	26 (47)	1 (5)	28 (25)
cnf1	0	0	25 (45)	0	25 (23)
ibeA	0	0	9 (16)	1 (5)	10 (9)
usp	1 (4)	0	42 (76)	5 (24)	48 (44)

Co-associations of toxin encoding genes

The toxin encoding genes *hlyA, cnf1* and *usp* were strongly co-associated (Tab. 3). These three genes often appeared concomitantly (19 of *hlyA* positive strains (68 %) possessed also *cnf1* and *usp* gene sequences). The *ibeA* gene was associated only with the *usp* gene, albeit this correlation was small (Pearson's correlation coefficient r = 0.296), it was statistically significant (P = 0.002) (Table 3).

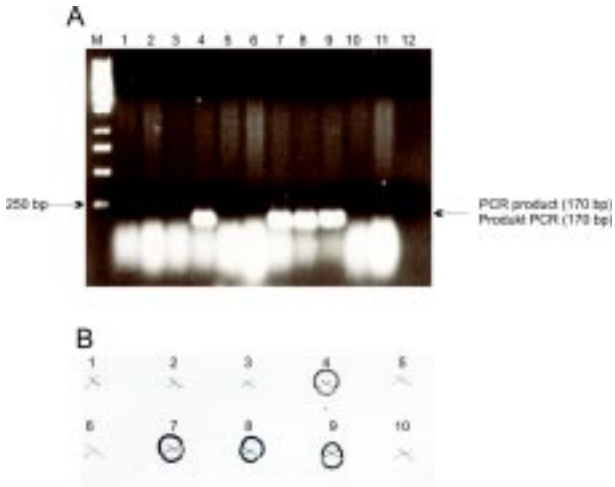

Figure 1. *An example of detection of toxin encoding genes – detection of the ibeA gene.*
(A) Visualization of PCR products obtained in PCR reactions on lysates of DL strains with primers specific for the ibeA gene (1 % agarose gel, stained with ethidium bromide).
M: marker –1 kb DNA ladder (Fermentas, Vilnius, Lithuania); 1: strain DL 3 (ibeA-); 2: strain DL 9 (ibeA-); 3: strain DL 23 (ibeA-); 4: strain DL 29 (ibeA+); 5: strain DL 30 (ibeA-); 6: strain DL 49 (ibeA-); 7: strain DL 54 (ibeA+); 8: strain DL 88 (ibeA+); 9: strain DL 89 (ibeA+); 10: strain DL 90 (ibeA-); 11: laboratory strain DH5α (ibeA-) and 12: negative control – a PCR reaction with sterile water instead of a lysate.
(B) Validation of the PCR assay with DIG hybridization of a ibeA specific probe on ibeA PCR products (10 µl) bound to a nylone membrane.
1: strain DL 3 (ibeA-); 2: strain DL 9 (ibeA-); 3: strain DL 23 (ibeA-); 4: strain DL 29 (ibeA+); 5: strain DL 30 (ibeA-); 6: strain DL 49 (ibeA-); 7: strain DL 54 (ibeA+); 8: strain DL 88 (ibeA+); 9: strain DL 89 (ibeA+); 10: strain DL 90 (ibeA-).

Sl. 1. *Primer detekcije genskega zapisa za toksin – detekcija gena ibeA.*
(A) Prikaz pridelkov PCR, dobljenih v reakcijah PCR na lizatih sevov DL z začetnimi oligonukleotidi specifičnimi za gen ibeA (1 % agarozni gel, obarvan z etidijevim bromidom).
M: standard – 1 kb DNK-lestvica (Fermentas, Vilnius, Litva); 1: sev DL 3 (ibeA-); 2: sev DL 9 (ibeA-); 3: sev DL 23 (ibeA-); 4: sev DL 29 (ibeA+); 5: sev DL 30 (ibeA-); 6: sev DL 49 (ibeA-); 7: sev DL 54 (ibeA+); 8: sev DL 88 (ibeA+); 9: sev DL 89 (ibeA+); 10: sev DL 90 (ibeA-); 11: laboratorijski sev DH5α (ibeA-) in 12: negativna kontrola – reakcija PCR s sterilno vodo, namesto lizata.
(B) Preverjanje PCR z DIG-hibridizacijo z vezavo sonde specifične za ibeA na produkte ibeA iz PCR (10 µl), vezane na najlonski membrani.
1: sev DL 3 (ibeA-); 2: sev DL 9 (ibeA-); 3: sev DL 23 (ibeA-); 4: sev DL 29 (ibeA+); 5: sev DL 30 (ibeA-); 6: sev DL 49 (ibeA-); 7: sev DL 54 (ibeA+); 8: sev DL 88 (ibeA+); 9: sev DL 89 (ibeA+); 10: sev DL 90 (ibeA-).

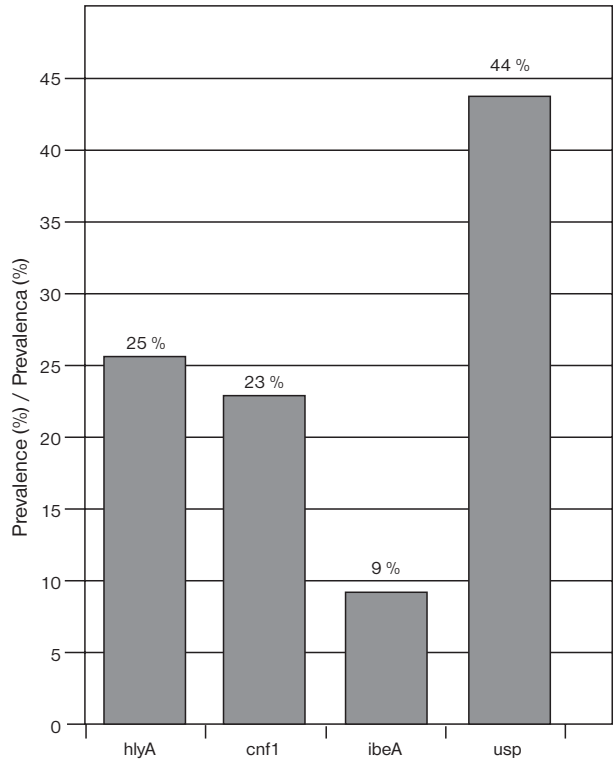

Figure 2. *Prevalence (in % of the total 110 studied E. coli strains) of tested toxin encoding gene sequences.*

Sl. 2. *Prevalenca (v % od 110 preučevanih sevov E. coli) genskih zapisov za toksine.*

Table 3. *Co-association of tested toxin encoding genes. Numbers below the diagonal are the r value of Pearson's correlation coefficient. Numbers above the diagonal are P values of Fisher's exact test.*

Razpr. 3. *Vezanost genskih zapisov za toksine. Števila pod diagonalo so vrednosti r Pearsonovega korelacijskega koeficienta. Števila nad diagonalo so vrednosti P Fisherjevega eksaktnega testa.*

Toxin encoding gene Genski zapis za toksin	*hlyA*	*cnf1*	*ibeA*	*usp*
hlyA		<0.001	NS/NZ	<0.001
cnf1	0.729		NS/NZ	<0.001
ibeA	0.040	0.021		0.002
ups	0.496	0.529	0.296	

NS/NZ = not significant / ni značilno

Discussion

The repertoire of virulence genes present in a certain strain determines the severity of disease.[4] Toxins are well established virulence factors that are often responsible for the major symptoms of a bacterial infection. In the presented study 110 UTI *E. coli* strains were characterized using PCR with primers specific for four toxin encoding genes: *hlyA*, *cnf1*, *ibeA* and *usp*. Analysis of the prevalence of *hlyA*, *cnf1*, *usp* and *ibeA* sequences among strains from our study (25, 23, 44, and 9 %, respectively) compared to studies of uropathogenic *E. coli* isolates from other geographic regions (Tab. 4) showed a comparable prevalence of

Table 4. *Comparison of results from different studies of UTI toxin encoding genes.*
Razpr. 4. *Primerjava rezultatov različnih raziskav genskih zapisov za toksine pri okužbi sečil.*

Study Raziskava	Toxin encoding gene prevalence (%) Prevalenca genskih zapisov za toksine (%)				Reference Referenca
	hlyA/D	*cnf1*	*ibeA*	*usp*	
76 pyelonephritis strains (Japan) 76 pielonefritičnih sevov (Japonska)	51	36	N	93	19, 20
74 cystitis strains (USA) 74 cistitičnih sevov (ZDA)	36	34	24	N	21
170 pyelonephritis strains (USA) 170 pielonefritičnih sevov (ZDA)	39	N	11	N	7
194 cystitis strains (Japan) 194 cistitičnih sevov (Japonska)	41	48	N	79	19, 20
100 cystitis strains (Israel) 100 cistitičnih sevov (Izrael)	44	34	N	N	22
78 UTI strains (Romania) 78 sevov iz okužb sečil (Romunija)	23	13	N	N	23
243 UTI strains (Italy) 243 sevov iz okužb sečil (Italija)	21	19	N	N	24
110 UTI strains (Slovenia) 110 sevov iz okužb sečil (Slovenija)	25	23	9	44	this study ta raziskava

N = not available / ni navedeno

toxin encoding genes. However, it has to be noted that among *E. coli* collections of strains causing either cystitis[19–22] or pyelonephritis[7, 19, 20] a higher prevalence of toxin encoding genes can be found than among collections of mixed UTI *E. coli* isolates.[23, 24, this study]
The tested toxin encoding genes were mostly found in strains belonging to the B2 phylogenetic group (Table 2). Picard et al.[25] established the link between phylogeny and extraintestinal virulence in the *E. coli* species. They showed that the strains of the B2 group represent a divergent lineage of highly virulent strains that possess the highest level of virulence determinants. Therefore the observed distribution of tested toxin encoding genes in our study is not surprising. Uropathogenic strains are known to carry large chromosomal regions, pathogenicity islands (PAI), encoding several virulence factors required for virulence. A number of PAIs have been defined in the archetypal uropathogenic strains 536, J96 and CFT073.[4] The high associations found between *hlyA, cnf1* and *usp* that are known to be carried on PAIs[4, 8] are therefore expected. When Marrs et al. were trying to define pathotypes of *E. coli* strains causing UTI, similar to pathotypes defined for *E. coli* isolates causing enteric/diarrheal diseases, they also found a strong association between *cnf1* and *hlyA* and these two genes were used as key genes to determine the two most virulent UTI pathotypes, pathotype 1 (*E. coli* isolates with HlyA, CNF1, class III P-fimbriae) and pathotype 2 (*E. coli* isolates with HlyA, CNF1, S-fimbriae).[26] Further, a statistically significant co-association of *ibeA* and *usp* was ascertained. To our knowledge in no previously published study all four toxin coding genes were screened for, so this is the first report of a statistically significant co-association of these two genes.
It should be noted, that some of UTIs have an organic cause, for example an obstruction of urinary tract in the form of urinary stones, or a tumor, which predisposes a patient to an infection. Further, the presentation of the infection is sometimes misapprehended as urinary tract irritation like urinary stone passage, overactive bladder or prostatism.[2] Therefore, further studies of the prevalence of toxin and other virulence factors encoding genes among UPEC strains isolated from patients with different clinical pictures would be interesting, especially as these results could build the basis for diagnostics of UPEC strains.
In summary, we have found among the UTI *E. coli* strains isolated in Slovenia a prevalence of common virulence toxin encoding genes comparable to those obtained in studies in different geographic regions.

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