The Distribution of Cytotoxic Necrotizing Factors (CNF-1, CNF-2, CNF-3) and Cytolethal Distending Toxins (CDT-1, CDT-2, CDT-3, CDT-4) in Escherichia coli Strains Isolated from Extraintestinal Infections and The Determination of Their Phylogenetic Relationship by PFGE

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Abstract

Background: Determinants of extraintestinal infection in *Escherichia coli* (*E. coli*) remain unclear. Virulence factors making Extraintestinal pathogenic *E. coli* (ExPEC) different from other *E. coli* strains are the host cell adhesion, invasion, and two important factors/toxins, Cytotoxic Necrotizing Factor (CNF) and Cytolethal Distending Toxin (CDT) that are responsible for cell death. In the present study, prevalence of CNF-CDT genotypes was investigated in 645 *E. coli* strains isolated from patients. This prevalence was analyzed regarding clinical origins, phylogroups and putative phylogenetic relationships.

Results: At least one virulence gene identified for ExPEC was found in 156 (24%) of 645 *E. coli* strains. 78, 12, 20 of ExPEC strains contained *cnf1*, *cnf2*, *cnf3*, respectively. Genes of *cdt1*, *cdt2*, *cdt3* and *cdt4* were detected as 20, 4, 4, 4. Finally, two factors were detected *cnf1*-*cnf3* (n=6), *cnf1*-*cdt1* (n=4), *cdt1*-*cdt4* (n=4). These 156 strains were found to be distributed in 106 large clusters by Pulsed-Field Gel Electrophoresis (PFGE). Virulent ExPEC was primarily related to the groups B2 (60%) and D (32%).

Conclusions: The CNF gene family is believed to enhance colonization of *E. coli* especially in the urinary system and the genes of *E. coli* gene pool gain the ability to survive in new environments, such as the human urinary tract. The widespread diffusion of the CNF gene in *E. coli* helps to distinguish ExPEC from commensal strains and these genes may be used as specific marker for non-commensal *E. coli*. It was concluded that different strains of *E. coli* gained ExPEC properties by acquiring different virulence genes resulting in deletion and recombination mutations, and it was thought that the infections caused by these strains were more likely community-acquired rather than hospital-acquired. Furthermore, in this study, *cnf2*, *cnf3*, *cdt1*, *cdt2*, *cdt3* and *cdt4* genes were demonstrated in Turkey for the first time.

Background

Escherichia coli (*E. coli*) is one of the major elements of the human colon microflora. *E. coli* can easily transfer virulence and resistance genes from other flora bacteria in the intestinal flora. Some geno-serotypes of *E. coli* cause pathologies in the intestinal tract, ranging from simple secretory diarrhea pathologies to inflammatory diarrhea in the form of dysentery with mortality. However, their colonization in sterile areas in the body, outside of the intestinal system, is always considered to be the cause of clinical pathology. Extraintestinal Pathogenic *E. coli* (ExPEC) causes urinary tract infection, newborn meningitis, sepsis, osteomyelitis, pneumonia, surgical site infections and infections in other extraintestinal areas. Although infections caused by ExPEC have low morbidity, it is becoming an increasingly important endemic problem due to its fatal course and prolonged hospital stay. Important virulence mechanisms that distinguish ExPEC from other *E. coli* strains include adhesion, invasion, defect of host defense and direct intervention in host cellular functions by secreted effectors (11, 12). These effectors are composed of a functional toxin class disrupting cell cycle, the basic cell process of the host cell. Especially toxins-factors in *E. coli* species are defined under 2 classes: Rho-GTPase-targeting toxin class is **cnf1-cnf3** (Cytotoxic Necrotizing Factor) and **cdt1-cdt4** (Cytolethal Distending Toxin), which is genotoxin. While CNFs promote DNA replication without cytokinesis, CDTs block mitosis (13, 14). These toxins-factors are encoded through mobile elements (island of pathogenicity, plasmids, bacteriophages, etc.) (15).

There have been a limited number of clinical-epidemiological studies revealing that ExPEC strains have different colonization and virulence factors and that polymorphism in genes encoding virulence factors affect the prognosis of the disease.

The aim of our study was to investigate the prevalence of CNF-CDT and the relationship between the clinical picture and genotypes in *E. coli* strains with different clinical origins. Thus, it was to detect a specific marker for the diagnosis of non-commensal *E. coli*.

Methods

Isolation and Identification

A total of 645 clinical samples (i.e., blood, urine, bronchoalveolar lavage, sputum, and surgical wound) were collected from different clinics of Balcalı Hospital at Cukurova University and of Adana Regional Hospital, Turkey, between September 2014 and April 2016. These 645 ExPEC strains were isolated from 387 outpatients and 129 inpatients. Most of the specimens were urine (80%) and most were taken from the polyclinics of urology and pediatric (Fig. 1-Figure 2). Other specimens taken in other medical units were wound, blood, sputum, aspirate, sterile body fluid, vagina and urosepsis (Fig. 1-Figure 2). The median age of patients with ExPEC was 45 years (range, new born to 82 years). While urine samples were obtained from 80 males and 442 females, other specimens including wound, blood etc. were obtained from 107 males and 16 females.

The samples were cultured on blood agar and Endo agar plates (Merck, Germany) at 37 °C for 24 hours. The isolates were first evaluated by Gram staining to examine the morphology of colonies and biochemical test characteristics. Strains were confirmed phenotypically by the IMVIC test and genotypically by the PCR method in the gene region (**uidA**) encoding Beta-D-glucuronidase enzyme found structurally in *E. coli* (Fig. 3). *E. coli* isolates were kept at -70 °C in broth containing 20% glycerol.

DNA Extraction and PCR

In all isolates, DNA extraction was performed mechanically with a Mickle cell disruptor (The Mickle Lab. Engineering Co. Ltd., Gamshall, Surrey, UK). The amplicons were obtained by PCR using previously published primers (Table 1) **uidA**, **cnf1**, **cnf2**, **cnf3**, **cdt1**, **cdt2**, **cdt3**, **cdt4** and extracted DNA samples. PCR amplifications were performed in a thermal cycler.

The PCR amplification was carried out in a total volume of 25 µl. The PCR mixture was constructed as follows: 12.5 µl PCR master mix (1x without MgCl₂, 4 µl MgCl₂ (25 mM), 1 µl dNTP (200 µM each nucleotides), 1 µl each primer, Taq DNA polymerase 0.2 µl U (5 U/µl), template DNA 5 µl (approximately 50 ng) and distilled water by completing to 25 microliters. The **uidA** gene and **cdt1**, **cdt2**, **cdt3**, **cdt4** genes were amplified in a single PCR reaction in one tube. Multiplex PCR protocol was used to determine **cnf1**, **cnf2**, **cnf3** genes. The PCR processes were applied for the amplifications of toxin genes (Table 2). The PCR products were separated on 2% agarose gel for **cdts** and **uidA**, and on 1.8% agarose gel for **cnfs**. Then, they were monitored using a Kodak UV transilluminator (Kodak, New York, USA).

Phylogenetic Classification

E. coli strains belonged to four groups (A, b1, b2, D) based on the presence of the **chuA** and **yjaA** genes and the DNA fragment (TSPE4.C2) (Table 1) by multiplex PCR as previously defined (Table 2). The results allowed the isolates to be classified into any of the four major phylogenies (224).

XbaI Pulsed field gel electrophoresis

PFGE was performed to determine DNA profiles of CNFs-CDTs producing strains. In our study, we applied the protocol described in a previous study (10) for *Klebsiella*, *E. coli*, *Paeruginosa*, *Acinetobacter* (KEPA), which are the most frequently isolated gram-negative bacilli from the clinical material. PFGE method was performed for the determination of the clonal association of ExPEC strains and as gold standard. Epidemiological relationships between strains were evaluated by studying the PFGE patterns of genomic DNA after restriction by *Xba*I. The *Xba*I-PFGE was performed as described in the previous study (10) (Fig. 4).

Table 1
Oligonucleotide primers used in the study

Primer	Target gene	Primer sequence (5'-3')	Fragment size (bp)	Reference
uidA	uidA-F	ATGCCAGTCCAGCGTTTTTGC	102	J. Abid, 2014
uidA	uidA-R	AAAGTGTGGGTCAATAATCAGGAAGTG	102	J. Abid, 2014
cnf1	cnf1-F	GGGGGAAAGTACAGAAGAATTA	1111	Dubois et al.,2010
cnf1	cnf1-R	TTGCCGTCCACTCTCACCAGT	1111	Dubois et al.,2010
cnf2	cnf2-F	TATCATACGGCAGGAGGAAGCACC	1240	Dubois et al.,2010
cnf2	cnf2-R	GTCACAATAGACAATAATTTTCCG	1240	Dubois et al.,2010
cnf3	cnf3-F	TAACGTAATTAGCAAAGA	757	Dubois et al.,2010
cnf3	cnf3-R	GTCTTCATTACTTACAGT	757	Dubois et al.,2010
cdt1	cdt1-F	CAATAGTCGCCCACAGGA	411	Toth et al., 2003
cdt1	cdt1-R	ATAATCAAGAACACCACCAC	411	Toth et al., 2003
cdt2	cdt2-F	GAAAGTAAATGGAATATAAATGTCCG	556	Toth et al., 2003
cdt2	cdt2-R	TTTGTGTTGCCGCCGCTGGTGAAA	556	Toth et al., 2003
cdt3	cdt3-F	GAAAGTAAATGGAATATAAATGTCCG	555	Toth et al., 2003
cdt3	cdt3-R	TTTGTGTCGGTGCAGCAGGGAAAA	555	Toth et al., 2003
cdt4	cdt4-F	CCTGATGGTTCAGGAGGCTGGTTC	350	Toth et al., 2003
cdt4	cdt4-R	TTGCTCCAGAATCTATACCT	350	Toth et al.,2003
chuA	chuA-F	GACGAACCA ACGGTCAGGAT	279	Clermont et al., 2010
chuA	chuA-R	TGCCGCCAGTACC AAAGACA	279	Clermont et al., 2010
yjaA	yjaA-F	TGAAGTGTCAGGAGACGCTG	211	Clermont et al., 2010
yjaA	yjaA-R	ATGGAGAATGCGTTCCTCAAC	211	Clermont et al., 2010
TspE4C2	TspE4C2-F	GAGTAATGTCGGGGCATTCA	152	Clermont et al., 2010
TspE4C2	TspE4C2-R	CGCGCCAACAAAGTATTACG	152	Clermont et al., 2010

Table 2
Thermal cycles for eleven primers used in the study

No	Step	uidA	cnfs	cdts	chuA, yjA, tspe4.c2
1	Initial denaturation	94 °C/5 min.	94 °C/3 min.	94 °C/5 min.	94 °C/5 min.
2	Denaturation	94 °C/30 sec.	94 °C/1 min.	94 °C/1 min.	94 °C/30 sec.
	Annealing	63 °C/30 sec.	60 °C/1 min.	60 °C/1 min.	60 °C/30 sec.
	Extension	72 °C/1.5 min.	72 °C/1 min.	72 °C/1 min.	72 °C/1.5 min.
3	Final extension	72 °C/5 min.	72 °C/7 min	72 °C/10 min	72 °C/5 min.
4	Keeping	+ 4 °C/ ∞	+ 4 °C/ ∞	+ 4 °C/ ∞	+ 4 °C/ ∞

Results

In the period of three years, a total of 645 ExPEC strains were studied. 522 of the samples were urine, 54, 28, 21, 6, 12, 3 and 1 of them were wound, blood, sputum, aspirate, sterile body fluid, vaginal and urosepsis, respectively (Fig. 1). In 156 (24%) of the 645 clinical samples, at least 1 virulence gene-positive was found. One hundred fourteen (18%) of these samples had only CNF (**cnf1**, **cnf2**, **cnf3** and **cnf1-cnf3** genes were identified in 78, 12, 20 and 6 of the samples, respectively) (Fig. 5), thirty-six (5,4%) had only CDT (**cdt1**, **cdt2**, **cdt3**, **cdt4** and **cdt1-cdt4** genes were identified in 20, 4, 4, 4 and 4 of the samples, respectively) (Fig. 6), and six (0,9%) had both toxins-factors) (Fig. 7) (Table 3).

According to the clinical origins of 645 ExPEC isolates, the distribution of virulence factors was found to be 3% in the ExPEC strains of the cdt gene family isolated from the urinary system and 15% in the non-urinary system isolates. While the rate of Cnf gene family was 20% in the urinary system, the ratio was 3% for the cdt gene family (Table 3).

Table 3
Distribution of Toxin Genes in 645 ExPEC strains

VFs (%)	All isolates (n = 645) n (%)	Urinary system n = 522 (80)	Non-urinary system							
			Total n = 123 (20)	Wound (n = 54)	Blood (n = 28)	Sputum (n = 21)	Aspirate (n = 6)	Sterile body fluid (n = 12)	Urosepsis (n = 3)	Vagen (n = 1)
cnf1	78(12)	68 (13)	10 (8)	3 (5)	3 (11)	3 (14)	1 (16)	-	-	-
cnf2	12 (2)	10 (2)	2 (1,6)	1 (2)	1 (4)	-	-	-	-	-
cnf3	20 (3)	16 (3)	4 (3,2)	2 (4)	1 (4)	1 (5)	-	-	-	-
cnf1-cnf3	6 (0,9)	5 (1)	1 (0,8)	-	1 (4)	-	-	-	-	-
Total cnfs	116 (18)	97 (20)	17(13)							
cdt1	20 (3)	12 (2)	8 (7)	-	1 (4)	2 (10)	2 (32)	2 (16)	1 (33)	-
cdt2	4 (0,6)	2 (0,3)	2 (1,7)	1 (2)	1 (4)	-	-	-	-	-
cdt3	4 (0,6)	2 (0,3)	2 (1,7)	-	-	1 (5)	-	-	-	-
cdt4	4 (0,6)	2 (0,3)	2 (1,7)	1 (2)	-	-	1 (16)	-	-	-
cdt1-cdt4	4 (0,6)	1 (0,2)	3 (3,2)	2 (4)	1 (4)	-	1 (16)	-	-	-
Total cdt	36 (5,4)	19 (3)	18 (15)							
cnf1-cdt1	4 (0,9)	3 (0,8)	1 (1,7)	1 (2)	-	-	-	-	-	-

In 156 *E. coli* isolates, at least one of whose virulence genes defined for ExPEC were positive and therefore can be identified as ExPEC (**cnf1**, **cnf2**, **cnf3-cdt1**, **cdt2**, **cdt3**, **cdt4**), the highest prevalence was **cnf1** among the virulence genes with a rate of 50% + 7.6% in binary combinations. This was followed by **cdt1** with a rate of 12.8% + 2.5% in binary combinations (Table 4).

Table 4
Distribution of Toxin Genes in CDT and CNF positive 156 ExPEC strains

Virulence factor	cnf1	cnf2	cnf3	cnf1-cnf3	cdt1	cdt2	cdt3	cdt4	cdt1-cdt4	cnf1-cdt1
Total (n = 156)	78	12	20	6	20	4	4	4	4	4
n/total	78/156	12/156	20/156	6/156	20/156	4/156	4/156	4/156	4/156	4/156
%	50	7,6	12,8	3,8	12,8	2,5	2,5	2,5	2,5	2,5

In the distribution of virulence genes / gene combinations detected in these strains via the clinical materials from which the strains were isolated, it was observed that 121 (78%) of 156 strains, where at least 1 virulence gene was positive, were associated with the urinary system, and the remaining 35 (22%) were isolated from extra-intestinal urinary infection foci. The most common of the virulence genotypes in 121 clinical materials originating from the urinary system was the **cnf** gene family with a rate of 78%. The **CDT** gene family was 17%. In **cnf-CDT** positive isolates, it was 2%. (Table 5).

While 68 of 78 **cnf1** positive strains originated from urinary system, 10 of them were isolated from other extraintestinal infection foci. Similar rates were observed in **cnf2** and **cnf3**. However, among the positive genes of **cdt1**, **cdt2**, **cdt3**, **cdt4**, only **cdt1** proved superiority in the urinary system with a rate of 10%, while **cdt2**, **cdt3** and **cdt4** were found in equal percentages. The **cdtB** gene family proved its superiority, especially in aspirated, sputum, peritoneal fluid, urosepsis and CSF material. The strains with at least 2 gene regions were found to be predominant in strains isolated from non-urinary tract infection. (Table 5).

Table 5

Distribution of factor-toxin genes of 156 ExPEC isolates by clinical origin / The virulence factor distributions in the urinary and non-urinary tract foci in **CDT** and **cnf** positive ExPEC strains

n(%) Urinary system n = 121 (78)		n(%) Non-urinary system n = 35 (22)						
Isolates	Urine (n = 121)	Wound (n = 11)	Blood (n = 9)	Sputum (n = 7)	Aspirate (n = 5)	CSF (n = 1)	Peritoneal fluid (n = 1)	Urosepsis (n = 1)
cnf1	68 (56)	3 (27)	3 (33)	3 (43)	1 (20)	-	-	-
cnf2	10 (8)	1 (9)	1 (11)	-	-	-	-	-
cnf3	16 (13)	2 (18)	1 (11)	1 (14)	-	-	-	-
cnf1- cnf3	5 (4)	-	1 (11)	-	-	-	-	-
cdt1	12 (10)	-	1 (11)	2 (29)	2 (40)	1	1	1
cdt2	2 (2)	1 (9)	1 (11)	-	-	-	-	-
cdt3	2 (2)	-	-	1 (14)	1 (20)	-	-	-
cdt4	2 (2)	1 (9)	-	-	1 (20)	-	-	-
cdt1- cdt4	1 (1)	2 (18)	1 (11)	-	-	-	-	-
cnf1- cdt1	3 (2)	1 (9)	-	-	-	-	-	-

Of the 156 ExPEC strains with at least one virulence gene, group b2 was the mostly associated with virulence with a rate of 60.8%. This was followed by group D at a rate of 30.7%. The rates of groups A and b1 were 5.12% and

3.2%, respectively (Table 6).

It was observed that **cnf1** was found to be significantly more frequent in the phylogroup b2 urinary tract strains than the phylogroup b2 non-urinary tract strains. While the same situation was valid for group D as well, the opposite was the case in group b1, and it was more common in non-urinary system isolates (Table 6).

Table 6
Distribution of 156 ExPEC isolates virulence genes by phylogenetic group and clinical origin

Virulence genes	No. (%) of virulence isolates (n = 156)		No (%) of isolates			
	Urinary/Others (n = 121)/(n = 35)		Group A n = 8 (5,1)	Group b1 n = 5 (3,2)	Group b2 n = 95 (60)	Group D n = 48 (30)
Urinary/Others	Urinary/Others		Urinary/Others	Urinary/Others		
cnf1	68 (56) 10 (29)		0 0	0 3 (60)	37 (39) 4 (4)	30 (62) 4 (8)
cnf2	10 (8) 2 (6)		3 (37) 0	0 0	7 (7) 2 (2)	0 0
cnf3	16 (13) 4 (11)		2 (25) 2 (25)	0 0	12 (13) 2 (2)	2 (4) 0
cdt1	12 (10) 8 (23)		0 1 (13)	0 0	12 (13) 7 (7)	0 0
cdt2	2 (2) 2 (6)		0 0	0 0	0 0	2 (4) 2 (4)
cdt3	2 (2) 2 (6)		0 0	0 0	0 0	2 (4) 2 (4)
cdt4	2 (2) 2 (6)		0 0	0 2 (40)	2 (2) 0	0 0
cnf1-cnfn3	5 (4) 1 (3)		0 0	0 0	5 (5) 1	0 0
cdt1-cdt4	1 (1) 3 (9)		0 0	0 0	1 (5) 3 (3)	0 0
cnf1-cdt1	2 (2) 2 (6)		0 0	0 0	0 0	2 (4) 2 (4)

With the XbaI-PFGE method, 156 strains determined to be ExPEC based on 80% similarity were collected in 106 clusters. It was revealed that four of the test strains (Z23, Z25, Z36, Z38) had 5 members, 8 of them (A, E, G, Z60, Z64, Z80, Z83 and Z88) had three members and 15 of them had two members (C, F, I, M, O, R, Z2, Z3, Z5, Z6, Z24, Z56, Z58, Z73 and Z76). In response to this, 81 strains were distributed into single-member clusters (Fig. 8).

The strains collected in a single-member cluster and in the same subset were determined to have different virulence factors. ExPEC isolates isolated from blood and urine clinical materials from the patients with urosepsis were found to be distributed in different clusters and had different virulence factors (for ExPEC strains, while **cdt1** was positive in the blood sample (Z), it was negative in the urine sample in terms of any virulence factors (Z10)) using the PFGE method (Fig. 9).

Discussion

ExPEC-related infections, which have a wide range of disease spectrum from urinary tract infections to deadly bacteremia, have increased from 17.8–65.3% in Turkey in the last 15 years (11). In this increase, it is important that ExPEC, which is under antibiotic pressure, gains new virulent properties with mobile genetic elements from different bacteria in order to survive under stress conditions. In addition to this, the use of irrational antibiotics is

responsible for that increase. With a limited number of clinical-epidemiological studies; direct intervention to host cell functions, damage to epithelial cells and potent virulence factors that can lead to tissue pathologies such as stimulating the inflammatory response have been demonstrated by CNF-CDT positivity.

CDTs are genotoxins causing DNA damage in target cells. In our study, we found the prevalence of CDT as 6.3% (40/645) (Table 3). While this rate is similar to 6.8% (222-223-9) having been reported in other studies; it is higher than the rate of 0.9%-2.5% (229–230). The variable prevalence of CDT genes can be explained by the fact that the test strains in our study and other studies were isolated from different extraintestinal infection areas.

CDT genes have been found to have a higher prevalence, especially in sepsis-related ExPEC strains compared to ExPEC strains isolated from other infection sites such as urinary tract infections (Table 3) (222-9). In our study, *cdt* gene was detected in 19 (3%) of 522 strains isolated from patients with urinary system infections and in 18 (15%) of 123 strains isolated from non-urinary system infections. The prevalence of urinary tract infections due to *E. coli* can also be explained by its being prone to easy colonization of the intestinal strains by invading into the urinary system through the neighborhood relationship and its capacity to easily create disease in the host under different stress factors without the need for an extra virulence factor. However, as a result of the disruption of the mucosal epithelial integrity of the mucosal epithelium in the intestinal lumen by CDT, which causes disruption of cellular integrity, ExPEC strains containing this factor easily translocate to the lamina propria prior to the intestines and then spreads to distant tissues with general circulation, making it possible to cause distant tissue infections - especially sepsis. This may explain why the prevalence of CDT is high in ExPEC strains isolated from patients with sepsis and pneumonia. Sequence analysis of the *E. coli* *cdt* operon indicated that this operon read *cdtA*, *cdtB* and *cdtC*; that is, it is necessary to read these 3 genes for the production of (231) active CDT. *CdtB* encodes enzymatically active protein subunits; polypeptides encoded by *cdtA* and *cdtC* are heterodimeric subunits required for binding of CDT to the target cell.

Four types of *cdtB*, which are defined as **cdt1**, **cdt2**, **cdt3**, **cdt4**, were identified in *E. coli*. (232). We detected all *cdtB* alleles in our study (Table 4). These findings revealed that in our ExPEC test strains, the prevalence of **cdt1** and **cdt4** was higher than the prevalence of **cdt2** and **cdt3** (Table 4). Similar to the other studies, the **cdt1** and **cdt4** alleles were found, but differently, the **cdt2** and **cdt3** alleles were not found (222, 223, 234, 229, 238). These findings imply that the **cdt1** and **cdt4** alleles of the CDT genes show very common and close homology and may have been derived from a common ancestor by phage transduction. Unlike these groups, it can be explained by many factors such as our finding that the **cdt2** and **cdt3** allo-types, namely the richness of our alleles, had local stress differences as well as the variety of our case groups in which we isolated the test strains.

On the other hand, CNF is the first toxin produced by the pathogen *E. coli* strains and reported to activate Rho GTPases dominantly. This toxin got this name because of its necrotizing effect on rabbit skin (134). We detected that 120 (18.9%) of the 645 *E. coli* isolates included in the study carried at least one *cnf* allele (Table 3). In previous studies, this rate has been between 5–34% and it can be asserted that the rates are similar to each other considering the number of test strains (240-241-242), and deviations may stem from unreported patient characteristics.

cnf1 is responsible for tissue damage and impairs epithelial barrier function in tissue culture systems (149,150) and affects the function of immune cells by blocking phagocytosis (151). **cnf1** induces the expression of cyclooxygenase-2 (COX-2), activates nuclear factor-kappa B (NF-κB), increases cell mobility and inhibits apoptosis. Strong evidence has been obtained that **cnf1** may play a role in cancer development (152–154). **cnf1** delete

mutants have been shown to have a lower potential to colonize the urinary system (148). In our study, 78 (65%) of the necro-toxigenic *E. coli* isolates, detected as 120, were **cnf1** positive. On the other hand, **cnf1** ratio was above 50% in 156 ExPEC isolates with positive virulence genes. In other studies, this rate has varied between 16.5% and 61.9% (143-243-244-229-222). In a study conducted in Turkey by Bozcal et al; **cnf1** ratio was 12% in ExPEC isolates isolated from blood samples (32). In our study, considering only blood samples, this rate was 11% (3/28) (Table 3) and it can be stated that the results are similar. These results indicated that **cnf1** may be a specific marker for virulent strains, especially for the urinary tract and sepsis-related ExPEC.

In our study, contrary to **cnf1** (56%), **cnf2** with a rate of 7,6% and **cnf3** with a rate of 16% were found less (Table 4). This can be explained by the fact that **cnf2** and **cnf3** are of animal origin and are rare in human beings. However, as opposed to other studies, prevalence of **cnf2** and **cnf3** was high in our study (222, 233, 241, 242, 243). This rate may represent the prevalence closer to reality, which was also due to the high number of strains.

Phylogenetic analysis revealed that natural *E. coli* isolates were divided into four main phylogenetic groups: A, B1, B2 and D. With using a simple and fast phylogenetic grouping technique triplex PCR (9), the group being mostly associated with virulence was B2 with a rate of 60% among 156 ExPEC strains having at least one virulence gene. This was followed by group D with a rate of 30%. The rates for the groups A and B1 were 5.1% and 3.2%, respectively (Table 6). Considering other studies, it can be claimed that ExPEC originated predominantly from phylogroup B2 and group D to a less extent (230-229-224-248-242-222). However, in the study carried out by Bozcal et al., these rates were D (38.14%), A (29.89%), B2 (20.61%) and B1 (11.34%) in ExPEC isolates isolated from blood samples. The reason for this difference may be the high number of samples in this study, the diversity of our clinical materials (such as urine, blood, wound, sputum, aspirate etc.) and different regions. In our study, in CNF-CDT positive strains; the PFGE method was used to reveal whether *E. coli* strains evolved from deletion, recombination mutations or evolved from an ancestor strain and acquired the ExPEC feature. In determining phylogenetic relationships of 156 strains known to be ExPEC, based on 80% similarity in the whole genome DNA fragment polymorphism analysis with XbaI-PFGE, it was observed that the test isolates were distributed in 106 large clusters, the largest of which was 5-membered (Fig. 8). ExPEC isolates with the same phylogenetic properties may have different virulence characteristics (Fig. 9). ExPEC isolates isolated from the same patient but from different clinical materials may originate from evolutionarily different ancestors. This may explain that most extra-intestinal *E. coli* infections were recovered from the community. Oloomi et al. (249) identified CNF-CDT (+) *E. coli* strains as highly heterogeneous. In a study conducted by Mora et al. (250) with 59 ExPEC strains, 85% similarity was taken as basis; 36 of the 59 strains were collected in 12 clusters; however, they found that 23 strains were distributed into single-member clusters and concluded that the XbaI-PFGE profiles of ExPEC isolates, which have recently been shown to move away from a common ancestor, form a homogeneous clonal group based on their significant similarities. Because of the redundancy in the number of clusters and the low rate of similarity between strains, our study may represent the heterogeneity which is closer to reality, resulting from the excess of our strain count.

Conclusion

The widespread diffusion of the *cnf* gene in *E. coli* can help to distinguish ExPEC from commensal strains and these genes can be a specific marker for non-commensal *E. coli*. Especially, *cnf-1* increases the *E. coli* colonization in the urinary system and it can be a characteristic virulence factor for the uropathogenic *E. coli* isolates. The CDT gene family support the colonization of *E. coli*, in extraintestinal areas outside the urinary system. Virulence factors

can be distributed depending on the phylogenetic groups (A, B1, B2, and D) and these phylogroups can be used in separation of pathogen-non pathogen.

Considering the XbaI-PFGE results, the phylogenetic relationships of ExPEC isolates were very weak, so it was inferred that *E. coli* strains can acquire ExPEC feature by acquiring different virulence genes as a result of deletion and recombination mutations under adverse conditions including antibiotic suppression. For this reason, multiple drug resistance may be common among ExPEC strains and this may result in treatment failure. Moreover, infections caused by these strains are more likely to be community-acquired rather than hospital-acquired. In addition, the fact that ExPEC isolates belonging to the same clusters display different virulence characteristics is another indication that these strains are of community origin. The characteristics and spread of virulent ExPEC strains should be monitored by molecular surveillance and limited to vaccine studies.

Abbreviations

ExPEC; Extraintestinal pathogenic *Escherichia coli*; PFGE: Pulsed-Field Gel Electrophoresis; CNF: Cytotoxic Necrotizing Factor; CDT: Cytolethal Distending Toxin.

Declarations

Ethics approval and consent to participate

This study was carried out with the approval of Çukurova University Faculty of Medicine Non-Interventional Clinical Research Ethics Committee (Date: 10.10.2014 and Decision No: 35-2).

Consent for publication

Not applicable.

Availability of data and materials

The datasets during and/or analysed during the current study available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

COG collected samples, cultured the isolates, and performed DNA extraction, PCR, PFGE. FK and COG designed the study. FK, COG and TN supervised the practical work and data management. COG and FK wrote the manuscript. All authors approved the final version of the manuscript. The authors were written in order.

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Figures

Figure 1

Distribution of isolates by clinical origin

Figure 1

Distribution of isolates by clinical origin

Figure 2

Distribution of 645 ExPEC isolates by Clinic and Polyclinics

Figure 2

Distribution of 645 ExPEC isolates by Clinic and Polyclinics

Figure 3

Gel image showing the band profile of the uidA genes region

Figure 3

Gel image showing the band profile of the uidA genes region

Figure 4

PFGE Resulting Band Profiles

Figure 4

PFGE Resulting Band Profiles

Figure 5

Gel image showing the band profile of the CNF genes region

Figure 5

Gel image showing the band profile of the CNF genes region

Figure 6

Gel image showing the band profile of the CDT genes region

Figure 6

Gel image showing the band profile of the CDT genes region

Figure 7

Gel image showing the band profile of the both toxin-factor genes region

Figure 7

Gel image showing the band profile of the both toxin-factor genes region

Figure 8

Dendrogram with PFGE Result

Figure 8

Dendrogram with PFGE Result

Figure 9

Clustering of virulence genotyping

Figure 9

Clustering of virulence genotyping

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