

The relation of phylogroups, serogroups, virulence factors and resistance pattern of *Escherichia coli* isolated from children with septicemia

F. Nojoomi and A. Ghasemian

1) Department of Microbiology, Faculty of Medicine, Aja University of Medical Sciences, Tehran, Iran

Abstract

The characterization of virulent and drug-resistant *Escherichia coli* strains helps to control and provide more accurate information regarding infection and eradication. The aim of this study was to determine the relationship between antibiotic susceptibility, phylogroups and virulence factors of *E. coli* isolates from children with septicaemia. One hundred dereplicated *E. coli* isolates were collected from paediatric patients with septicaemia in five hospitals in Tehran (May 2015 to May 2018). The antibiotic susceptibility of isolates was performed as per the 2016 guidelines of the Clinical and Laboratory Standards Institute. Extended-spectrum β -lactamases and carbapenemase genes, phylogroups, serogroups and virulence encoding genes were detected by PCR. Phylogroup B2 was dominant (40%) among strains, followed by phylogroups D (30%), A (8%) and B1 (7%). CTX-M1 was significantly higher in the B2 group ($n = 21$, $p = 0.001$). Furthermore, the virulence genes *iutA* ($n = 27$, $p = 0.002$), *csgA* ($n = 39$, $p < 0.001$), *kpsMIII* ($n = 39$, $p = 0.002$), *ibeA* ($n = 4$, $p = 0.004$), *vat* ($n = 5$, $p = 0.003$), *traT* ($n = 24$, $p < 0.001$), *sat* ($n = 12$, $p = 0.001$) and *hlyA* ($n = 33$, $p < 0.001$) showed significantly higher rates in phylogroup B2. Three O25/CTXMI/OXA-48 and *cnf*, *iutA*, *csgA* and *traT* positive isolates belonged to phylogroup B2. Pulsed-field gel electrophoresis analysis showed 85% similarity among 25% of isolates. More than half of the isolates were multidrug-resistant *E. coli*. A significant relation was observed among *iutA*, *csgA*, *kpsMIII*, *ibeA*, *vat*, *traT*, *sat* and *hlyA* genes and phylogroup B2. The characterization of virulent and drug-resistant strains helps control and properly eliminate infections. There was no genetic relation among strains in the pulsed-field gel electrophoresis pattern. © 2019 Published by Elsevier Ltd.

Keywords: Drug resistance, *Escherichia coli*, phylogenetic analysis, virulence

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Corresponding author: F. Nojoomi, Department of Microbiology, Faculty of medicine, Aja University of medical sciences, Tehran, Iran
E-mail: fnojoomi2@gmail.com

Introduction

Escherichia coli is among the dominant bacterial species causing septicaemia, pyelonephritis, urinary tract infections (UTIs), cystitis and travellers' diarrhoea, as well as meningitis in newborns [1]. *E. coli* is the most common organism which causes gastrointestinal, urinary tract and extraintestinal infections (ExPEC) [2]. Sepsis is a potentially life-threatening clinical infection associated with bacterial organisms, mainly due to Gram-negative species, and *E. coli* is the most common Gram-

negative agent [3]. The body's response to sepsis or bacteraemia leads to septic shock due to the development of disease and to conditions that could lead to a high mortality rate among children [4–6]. Patients with sepsis must be promptly diagnosed because the disease is possibly preventable [7]. The most common infection before sepsis is UTI [8]. Septicaemic infections develop after UTI and genital infections in all age groups.

Phylogenetic analyses have determined that *E. coli* strains may be placed in four major phylogenetic groups, including A, B1, B2 and D types. The relationship between phylogenetic groups and virulence factors has not been fully proven [9,10]. ExPEC infections mainly belong to the B2 and D phylogroups [11]. Furthermore, antibiotic resistance has increased among *E. coli* strains, and the worldwide spread of some virulent clonal complexes such as ST151 have restricted chemotherapy options [12,13]. ExPEC strains contain a variety of virulence

factors such as α -haemolysin (*hly*), cytotoxic necrotizing factor (*cnf*), invasion of endothelial brain protein (*ibeA*), fimbriae curli (*csgA*), toxin-secreted autotransporter (*sat*), serine protease autotransporter (*pic*), vacuolization of toxin autotransporter (*vat*), amyloid protein associated with serum resistance (*traT*) and capsule K1 (*kpsMTII*) [14,15]. The antigenic structures of strains can be detected with molecular methods such as PCR. ExPEC causing septicæmia mainly belong to one of 12 common O antigens including O1, O2, O4, O6, O7, O12, O15, O16, O18, O25, O75 and O157 [16,17].

The aim of this study was to determine the relationship between antibiotic resistance and serogroups and virulence factors of *E. coli* isolates among children with septicæmia.

Materials and Methods

Clinical isolates

We studied 456 dereplicated ExPEC *E. coli* isolates from various age groups (age range, 8 months to 89 years), identified using phenotypic and biochemical tests (blood agar, MacConkey agar, citrate, indole, motility) from blood samples among children with septicæmia in five hospitals of Tehran, Iran, during May 2015 to May 2018. One hundred of them were from patients aged 8 months to 11 years. No patient aged 12 to 15 years old was included in the study; the exclusion criterion was children aged >12 years. We also excluded isolates from gastrointestinal infections. The isolates were identified by biochemical tests and were next preserved in trypticase soy broth and 30% glycerol at -20°C .

Antibiotic susceptibility testing

Antibiotic susceptibility testing was implemented with the disc diffusion method (Kirby-Bauer) as per 2016 Clinical and Laboratory Standards Institute (CLSI) recommendations using 15 antibiotics, including fosfomicin (200 μg), ceftazidime (30 μg), co-amoxiclav (30 μg), ceftazidime (30 μg), cefotaxime (30 μg), erythromycin (15 μg), nitrofurantoin (300 μg), gentamicin (10 μg), tetracycline (30 μg), piperacillin/tazobactam (110 μg), amoxicillin (25 μg), meropenem (10 μg), imipenem (10 μg), tigecycline (25 μg) and ciprofloxacin (5 μg). *E. coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 strains were used as the quality control of the discs in susceptibility testing.

Phenotypic extended-spectrum β -lactamase (ESBL) production was confirmed with synergy tests (using co-amoxiclav and ceftazidime/cefotaxime discs) and Etest according to CLSI guidelines. Additionally, the combined discs including imipenem with ethylenediaminetetraacetic acid (EDTA) and the Carba-NP test were performed for determination of carbapenemase production.

DNA isolation and PCR technique

Isolation of bacterial DNA was implemented with the boiling method as described elsewhere [18,19]. The PCR reaction was used to amplify phylogenetic sequences, serogroups and virulence genes.

Phylogenetic typing

Phylogenetic grouping was conducted as C2 by PCR according to the *chuA* and *yjaA* genes and the DNA fragment TspE4 as described by Clermont *et al* [20]. The primer pairs were ChuA F-(5'-GACGAACCAACGGTCAGGAT-3'), R-(5'-TGCCGCC AGTACCAAAGACA-3'), YjaA F-(5'-TGAAGGTCAGGAGAC GCTG-3'), R-(5'-ATGGAGAATGCGTTCCTCAAC-3') and TspE4C2 F-(5'-GAGTAATGTCGGGGCATTCA-3'), R-(5'-CGCGCCAACAAAG TATTACG-3'). The PCR conditions were as follows: 4 minutes at 94°C ; 30 seconds, 33 cycles of 45 seconds at 94°C , 45 seconds at 55°C and 45 seconds at 72°C ; and a final extension step of 10 minutes at 72°C .

O serogroup amplification

The specific primers and thermal profiles we used are presented in Table 1. PCR conditions included 5 minutes at 94°C ; 30 cycles of 30 seconds at 94°C , 1 minute at 61°C and 50 seconds at 72°C ; and 10 minutes at 72°C .

Virulence typing

Virulence genes including cytolethal distending toxin (*cdt*), capsular polysaccharide synthesis K1 (*kpsMTII*), receptor Toll/interleukin 1 (*TcpC*), cytotoxic necrotizing factor 1 (*cnf1*), ferric aerobactin receptor (iron uptake/transport) (*iutA*), serum survival (*traT*), invasion of brain endothelium (*ibeA*), α -haemolysin (*hlyA*), surface exclusion, curli fimbriae (*CsgA*), secretion auto-inducer toxin (*sat*), vaculating autoinducer toxin (*vat*) and serine protease autoinducer (*pic*) were amplified by PCR using the specific primers listed in Table 2.

Pulsed-field gel electrophoresis genotyping

Pulsed-field gel electrophoresis (PFGE) was performed as previously described [27]. In brief, equal volumes of the standardized bacterial suspensions ($\text{OD}_{610} = 0.6$) and 1% Seakem Gold agarose were mixed gently and allowed to solidify into agarose plugs. The plugs were lysed with cell lysis buffer (50 mM Tris, 50 mM EDTA (pH = 8), 1% sarcosine, 1 mg/mL proteinase K; Promega, Madison, WI, USA) and incubated for 4 hours at 54°C . Then lysed plugs were washed two times with ddH_2O and six times with TE buffer. The plugs were digested with 10 U of *XbaI* (Promega) for 24 hours at 37°C . DNAs were separated by using a CHEF-Mapper with pulse times of 2.25 to 54.2 seconds at 200 V for 24 hours [28]. Analysis of the PFGE banding patterns based on the unweighted pair-group method

TABLE 1. Primers used for amplification of O serogroups [20]

Primer	Sequence	Product size (bp)
gndbis.F	5'-ATACCGACGACGCCGATCTG-3'	—
rfbO1.R	5'-CCAGAAATACACTTGGAGAC-3'	189
rfbO2.R	5'-GTGACTATTTTCGTTACAAGC-3'	274
rfbO4.R	5'-AGGGGCCATTTGACCCACTC-3'	193
rfbO6.R	5'-AAATGAGCGCCACCACTTAC-3'	584
rfbO7.R	5'-CGAAGATCATCCACGATCCG-3'	722
rfbO12.R	5'-GTGTCAAATGCCTGTCACCG-3'	239
rfbO15.R	5'-TGATAATGACCAACTCGACG-3'	536
rfbO16.R	5'-GGATCATTATGCTGGTACG-3'	450
rfbO18.R	5'-GAAGATGGCTATAATGGTTG-3'	360
rfbO25.R	5'-GAGATCCAAAAACAGTTTGTG-3'	313
rfbO75.R	5'-GTAATAATGCTTGCAGAAACC-3'	419
rfbO157.R	5'-TACGACAGAGAGTGTCTGAG-3'	672

was carried out by BioNumerics 6.0 software with arithmetic averages with 1.5 position tolerance.

Statistical data

Data were analysed by SPSS 21 (IBM, Armonk, NY, USA). The t test (chi-square analysis) and F test (one-way ANOVA) were done for this purpose. The 95% confidence interval was optimized, and p < 0.05 was considered a significant result.

Ethical approval

This study was ethically approved by AJA University of Medical Sciences.

Results

Demographic data of patients

Paediatric patients included 32 boys (mean age of 6 years) and 68 girls (mean age of 7 years). Twenty per cent of children (12 boys and eight girls) were diagnosed with both septicaemia and UTI at the same time. Hospital wards included intensive care unit (24%), haematology (13%), urology and nephrology (21%), renal unit (12%), emergency clinic (11%), cardiac care unit (5%), gastroenterology (8%), cardiology and rheumatology (5%) and pulmonary/respiratory diseases (4%). Previous hospitalization during 2 months or more was observed among 87 of 100 children (p 0.0013).

Susceptibility pattern

The majority of isolates were resistant to erythromycin (89%), tetracycline (86%) and amoxicillin (80%). However, all were susceptible to fosfomycin and nitrofurantoin, and the majority was susceptible to imipenem, meropenem (85% for both) and piperacillin/tazobactam (60%). Furthermore, 57%, 14%, 33%, 71%, 48%, 56%, 24% and 49% were resistant to ceftazidime, amikacin, gentamicin, ciprofloxacin, co-amoxiclav, cefotaxime, tigecycline and nalidixic acid respectively. Fifty-five per cent of isolates exhibited resistance to three classes of antibiotics (erythromycin, tetracycline and ceftazidime), indicating multidrug-resistant (MDR) *E. coli*.

TABLE 2. Primers and used for detection of virulence genes

Primer	Primer direction	PCR product size (bp)	Reference
<i>cdt</i>	F: 5'-AAATCACCAAGAATCATCCAGTTA-3'	430	[21]
	R: 5'-AAATCTCCTGCAATCATCCAGTTA-3'		
<i>kpsMIII</i>	F: 5'-GCGCATTGCTGATACTGTTG-3'	272	[21]
	R: 5'-CATCCAGACGATAAGCATGAGCA-3'		
<i>TcpC</i>	F: 5'-GAGTGGAAAGGAGTTGAGGC-3'	544	[22]
	R: 5'-GCAGTGCCATTTTATCCGCC-3'		
<i>iutA</i>	F: 5'-GGCTGGACATCATGGGAACTGG-3'	302	[23]
	R: 5'-CGTCGGGAACGGGTAGAACG-3'		
<i>traT</i>	F: 5'-GGTGTGGTCCGATGAGCACAG-3'	290	[23]
	R: 5'-CACGGTTCAGCCATCCCTGAG-3'		
<i>hlyA</i>	F: 5'-GCATCATCAAGCGTACGTTCC-3'	534	[24]
	R: 5'-AATGAGCCAAGCTGGTTAAGCT-3'		
<i>cnfI</i>	F: 5'-AAGATGGAGTTTCTATGCGAGAG-3'	498	[25]
	R: 5'-CATTGAGAGTCTGCGCCTCATTAT-3'		
<i>ibeA</i>	F: 5'-AGGCAGGTGTGCGCCGCGTAC-3'	171	[25]
	R: 5'-TGGTCTCCGGCAAACCATGC-3'		
<i>vat</i>	F: 5'-AACGGTTGGTGGCAACAATCC-3'	420	[25]
	R: 5'-AGCCCTGTAGAATGGCGAGTA-3'		
<i>sat</i>	F: 5'-TCAGAAGCTCAGCGAATCATTG-3'	930	[25]
	R: 5'-CCATTATCACCAGTAAAAACGCC-3'		
<i>pic</i>	F: 5'-ACTGGATCTTAAGGCTCAGGAT-3'	572	[25]
	R: 5'-GACTTAATGTCACTGTTGAGCC-3'		
<i>CsgA</i>	F: 5'-GGCGAAATGGTTCAGATGTTG-3'	295	[26]
	R: 5'-CGTATTCATAAGCTTCTCCCGA-3'		

Of 57 ceftazidime-resistant *E. coli*, 46 were ESBL producers. In the combined discs including imipenem with ethylenediaminetetraacetic acid (EDTA) and Carba-NP test, among imipenem-resistant strains, 14 produced carbapenemases phenotypically.

PCR amplification of genes

Eight strains belonged to phylogroup A. All of them amplified *csgA*, *traT* and *hlyA* virulence genes. Six of these strains harboured the *bla*_{CTX-M1} β-lactamase gene. Moreover, four of the strains carried both the *sat* and *iutA* genes (Table 3). In addition, seven strains were classified as phylogroup B1, and all were CTX-M1 positive. Five of them amplified the O15 and O75 serogroups. All of strains which were O75 positive also contained *iutA*, *csgA* and *KpsMIII* virulence genes. Two children had the *vat* gene and had a history of antibiotic therapy in the past 2 months. Phylogroup B2 was dominant (40%) among strains from children. Sixty per cent of them belonged to serogroups O25, O18 and O75. All these strains amplified *cnf*, *ibeA* and *sat* virulence genes. Moreover, *iutA*, *csgA*, *kpsMIII* and *hlyA* were dominant virulence genes among strains in phylogroup B2. Fourteen of the children had a history of antibiotics therapy in the past 2 months.

Thirty per cent of isolates classified in phylogroup D predominantly belonged to the O1 (n = 24) and O2 (n = 3) serogroups. Sixteen patients showed liver and kidney disorders. In this phylogroup, *iutA*, *traT*, *csgA*, *KpsMIII* and *hlyA* genes were predominant (Table 3). Two isolates could amplify the *pic* gene. Moreover, two strains belonged to serogroup O75 and were CTX-M1 positive. The virulence genes *tcpC* and *cdt*, and *Klebsiella pneumoniae* carbapenemase (KPC) and Verona

TABLE 3. Statistical relation of *Escherichia coli* phylotypes and characteristics of isolates

Encoding gene	Phylogroup A (n = 8)	Phylogroup B1 (n = 7)	Phylogroup B2 (n = 40)	Phylogroup D (n = 30)	p
SHV	4	2	9	7	0.455
CTX-M1	6	7	21	4	0.001
TEM1	2	2	5	2	0.022
OXA-48	0	0	1	2	0.240
VIM	0	0	0	0	—
IMP	1	0	2	1	0.240
KPC	0	0	0	0	—
<i>cnf</i>	2	1	10	1	0.001
<i>cdt</i>	0	0	0	0	—
<i>iutA</i>	7	7	27	20	0.002
<i>csgA</i>	8	1	39	30	<0.001
<i>kpsMII</i>	7	7	39	28	0.002
<i>ibeA</i>	0	0	4	3	0.004
<i>vat</i>	0	2	5	3	0.003
<i>traT</i>	8	1	24	28	<0.001
<i>sat</i>	0	1	12	9	0.001
<i>pic</i>	0	0	0	2	0.014
<i>hlyA</i>	8	2	33	22	<0.001

integron-encoded metallo- β -lactamase (VIM) β -lactamase were not detected among the phylogroups.

The prevalence of *cnf*, *cdt*, *iutA*, *csgA*, *kpsMII*, *ibeA*, *vat*, *traT*, *sat*, *pic* and *hlyA* were 11%, 0, 61%, 86%, 66%, 14%, 14%, 81%, 28%, 4% and 74% respectively. The prevalence of SHV, TEM1, CTX-M1, OXA-48 and IMP were 13%, 11%, 38%, 3% and 4% respectively. None of them carried the KPC or the VIM genes.

The O1 and O75 serogroups were predominant (each 20%), followed by O2, O18, O25, O4, O6, O7, O12, O15, O16 and O157, at 10%, 10%, 7%, 5%, 5%, 5%, 0, 5%, 3% and 0 respectively. Ten per cent of isolates were not typed with serogroup amplification in this study. Three isolates were O25/CTXM1/OXA48 positive, which encoded *cnf*, *iutA*, *csgA* and *traT* and belonged to phylogroup B2.

A comparative study was performed between children ($n = 100$) and adults ($n = 356$) with regard to phylogroups, O1 and O75 serogroups, MDR strains and each of virulence genes. It is noteworthy that only MDR strains were significantly higher among adults, but none of the factors was significantly different. The existence of *bla*_{CTX-M1} (72% vs. 38%, $p = 0.0111$), *bla*_{IMP} (27.80% vs. 4%, $p < 0.001$) and *bla*_{OXA-48-like} (21% vs. 3%, $p < 0.001$) genes were significantly higher among adults. Therefore, a higher rate of drug resistance was observed among adults compared to children.

PFGE analysis

PFGE typing of 100 isolates from paediatric patients revealed that 95 various types were classified. Twenty-five isolates (25%) demonstrated $\geq 85\%$ similarity as analysed by PFGE.

Discussion

In this study, most of the *E. coli* strains that caused septicaemia in paediatric patients genetically showed a high-level virulence

and carried several virulence genes. We found that among paediatric patients with septicaemia, there was a higher prevalence of the *csgA*, *traT*, *iutA*, *hlyA* and *kpsMII* genes. We also showed that phylogroup B2 was predominant among the isolates we studied here. This reveals that *E. coli* virulence determinants such as curli fimbriae, siderophore, capsule, α -haemolysin and serum amyloid protein associated with resistance play crucial roles in the colonization and invasion of the blood by the isolates. One study of hospitalized patients found that the α -haemolysin gene was an important factor leading to the urosepsis by *E. coli*, and similar to our results, the B2 phylogroup was the predominant phylogroup determined among these patients [29]. In a study from India, the dominant virulence gene among hospitalized patients was *hly*, and phylogenetic group B2 was the predominant type, similar to our results [30]. The high prevalence of the *hlyA* gene in this study suggests a possible relation between septicaemia and urinary tract isolates. Curli pili mostly contribute to the infection processes by providing the bacterial attachment structure, invasion to the host cells, spread to other organs and promotion of biofilm formation [31]. In this study, it was demonstrated that 86% of *E. coli* causing septicaemia in paediatric patients amplified the curli fimbriae (*csgA*) gene. Two other important virulence genes in this study were *kpsMII* (66%) and *traT* (81%). The *traT* and *iutA* had high prevalence among *E. coli* isolates in China (75.8% for *traT*) [32] and India (68% for *iutA*) [33], similar to this study, but 23% of them amplified *hlyA* and *cnfI* in India. The role of *traT* in *E. coli* isolates from blood in paediatric patients with septicaemia and bacteraemia has been demonstrated. The prevalence of *traT* has been indicated to have a relation to the expression of the K1 capsule [34]. Therefore, it may be suggested that *traT* and *kpsMII* with the *csgA* genes are major causes of sepsis among *E. coli* isolates from paediatric patients in Tehran hospitals. There was a significant relation between these virulence genes and phylogroup B2.

It was found that 40% of strains isolated from paediatric patients possessed O1 and O75 antigens. In this study, among the 12 most common *E. coli* O-antigen serogroups, the O1 and O75 antigens were more commonly detected among paediatric patients with septicaemia. The *E. coli* O1 serogroup mostly expresses the capsular K antigens and thus cause extraintestinal infections in humans [35]. In our study, the prevalence of O1/K antigenic serogroups was high; however, this serogroup is the main causative factor leading to UTIs and sepsis infection of paediatric patients.

Furthermore, among isolates with resistance to both ceftazidime and imipenem, the prevalence of SHV, TEM1, CTX-M1, OXA-48 and IMP were 13%, 11%, 38%, 3% and 4% respectively. Similar to these results, CTX-M1 is the predominant gene among ESBL-producing *E. coli* isolates worldwide. None of them could amplify the *KPC* and *VIM* genes. There was a significant relation between the presence of CTX-M1 and phylogroup B2. Receipt of antibiotic therapy in 2 past months (p 0.032) and liver and kidney disorders (p 0.001) were significant risk factors for MDR *E. coli* acquisition in paediatric patients. Genetic analysis of the isolates showed no clonal spread of *E. coli* causing septicaemia among children. We observed a higher rate of drug resistance and the presence of ESBL- and carbapenemase-encoding genes among isolates from adults compared to children. Additionally, none of the total 456 isolates carried the *KPC* or *VIM* genes.

In conclusion, it was found that approximately half of *E. coli* isolated from paediatric patients with septicaemia belonged to phylogroup B2, and half also carried O1 and O75 antigens. The majority of the isolates carried *csfA*, *kpsMII*, *iutA* and *traT* virulence factors; moreover, a significant relation was found between these genes and phylogroup B2. More than half of the isolates were MDR *E. coli*. The characterization of virulent and drug-resistant strains contributes to the control and proper therapy of infections. Genetic analysis by PFGE showed no clonal spread of *E. coli* isolates among paediatric patients. However, previous hospitalization was a significant risk factor in children, which should be considered with regard to the acquisition of nosocomial infections. It is noteworthy that there was no significant difference regarding phylogroups, serogroups and virulence factors between ExPEC from children and adults, but drug resistance and the existence of ESBL and carbapenemase genes were significantly higher among adults, thereby highlighting a higher rate of drug resistance among adults.

Conflict of Interest

None declared.

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