JIDC | THE JOURNAL OF INFECTION IN DEVELOPING COUNTRIES

Original Article

Characterization of diarrheagenic *Escherichia coli* strains associated with diarrhea in children, Khouzestan, Iran

Sanaz Mahdavi Borujerdi[1], Mohammad Roayaei Ardakani[1,2], Seyedeh Elham Rezatofighi[1,2]

[1] *Department of Biology, Faculty of Science, Shahid Chamran University of Ahvaz, Ahvaz, Iran*
[2] *Biotechnology and Biological Science Research Center, Shahid Chamran University of Ahvaz, Ahvaz, Iran*

Abstract

Introduction: Diarrheagenic *Escherichia coli* (DEC) is a major etiologic agent among the pathogens that cause diarrhea in children.

Methodology: To investigate the presence and pathotypes of DEC in children under five years of age, living in the province of Khouzestan, Iran. 208 diarrhea stool samples were screened by multiplex-PCR. The isolated DEC isolates were investigated for resistance to various antimicrobials including the production of extended-spectrum beta-lactamases (ESBLs) and phylogenetic groups were determined.

Results: DEC isolates were identified in 54 (26%) diarrhea samples, and 4 (7%) cases contained two DEC pathotypes. DEC isolated included 35 (16.8%) enteroaggregative *E. coli* (EAEC), ten (4.8%) enteropathogenic *E. coli* (EPEC), six (2.9%) enteroinvasive *E. coli* (EIEC), six (2.9%) enterotoxigenic *E. coli* (ETEC) and one (0.48%) LEE-positive EAEC. Shiga-toxin producing *E. coli* (STEC) was not identified in any diarrheal samples. The most prevalent resistance was observed with ceftazidime (88%), followed by ceftizoxime (83%) and ceftriaxone (71%). The majority of isolates (> 75%) were sensitive to Imipenem, ciprofloxacin, and amikacin. More than 65% of the pathogenic isolates showed a multidrug-resistant phenotype. ESBL-producing strains was observed in 79.3% of all DEC isolates. Phylogenetic group B2 was the most predominant group with a frequency of 44.8%. A significant association was observed between the B2 phylogenetic group and the DEC isolates ($P < 0.05$).

Conclusions: Overall, our findings highlight the importance of the role of DEC isolates in the etiology of diarrhea in children in Iran. The progressive increase in antimicrobial resistance among DEC isolates makes it imperative to implement policies to control the spread of resistant bacteria.

Key words: Diarrheagenic *Escherichia coli*; diarrhea; antimicrobial resistance; ESBL; phylogenetic group.

J Infect Dev Ctries 2018; 12(8):649-656. doi:10.3855/jidc.9538

(Received 23 June 2017 – Accepted 22 January 2018)

Copyright © 2018 Mahdavi Borujerdi *et al*. This is an open-access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Introduction

Gastrointestinal pathogens continue to be one of the major causes of morbidity and mortality affecting children under five years old in developing countries [1]. A wide range of bacteria, viruses, and parasites are the etiological agents of diarrhea. Among the bacteria, diarrheagenic *Escherichia coli* (DEC) is one the most important agents of endemic and epidemic diarrhea worldwide [2]. DEC isolates can be subdivided into at least six pathotypes according to pathogenic mechanisms including enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC) or shiga-toxin producing *E. coli* (STEC), enteroaggregative *E. coli* (EAEC) and diffusely adherent *E. coli* (DAEC) [3]. The molecular detection and classification of DEC is performed using polymerase chain reaction (PCR), based on the presence of virulence genes that are absent in commensal *E. coli* [4]. The prevalence and epidemiology of DEC pathotypes vary in different parts of the world and even within the in the same geographical area [5]. Understanding the etiological agents of diarrhea can influence local treatment policies for infectious diarrhea due to different pathotypes requiring different treatment regimes. Previous studies have shown the prevalence of DEC pathotypes to be highly variable in different parts of Iran. Aslani *et al.* reported that STEC is the most prevalent pathotype in Iran [6], while Alikhani *et al.* reported that EPEC is the most prevalent pathotype in Hamedan, Western Iran [7]. However, in studies conducted in Tehran (Central Iran) [8] and Tabriz (northwest Iran) [9], EAEC and ETEC were the most prevalent pathotypes, respectively. EHEC was detected in children [8] and animal [10] in some regions of Iran; although it′s prevalence was low. These studies show that the prevalence of DEC pathotypes is variable across Iran but southwest Iran has not been studied.

Antibiotic therapy may be used to combat with gastrointestinal infection, however, antimicrobial resistance has become a major public health problem [1]. Regular surveillance of antimicrobial resistance patterns provides information to enable appropriate clinical treatment and control the spread of resistance [6]. Antibiotic resistance surveillance is important with the discovery of multi-drug resistant (MDR) *E. coli* strains with extended-spectrum β-lactamases (ESBLs) that are widely distributed throughout the world [11]. ESBLs are β -lactamases that hydrolyze β-lactam antimicrobials, including penicillin, aztreonam, and first, second and third-generation cephalosporins, and are generally inhibited by clavulanic acid (β-lactamase inhibitors) [11]. These enzymes are capable of conferring resistance to bacteria, posing a therapeutic challenge [11]. The prevalence of ESBL-producing bacteria is extremely underestimated, and therefore early detection is incredibly important. In case of severe infection in children or infants, beta-lactams or quinolones are used in Iran. ESBL-producing bacteria play a very important role in the spread of antibiotic resistance.

E. coli strains are genetically diverse, and fall into four main phylogenetic groups: A, B1, B2, and D. The determination of a phylogenetic group allows physicians to understand the pathogenic potential of the organism including possible complications [12]. Commensal *E. coli* strains are more likely to fall into the A and B1 phylogenetic groups, while the extra intestinal pathotypes are more related to the B2 and D groups. However, there is little research about the phylogenetic grouping of DEC strains in association with antibiotic resistance [13], although a previous report described that the prevalence of antibacterial resistance was more likely to be found in non-B2 phylo-group *E. coli* strains [14]. There is also insufficient data in the association of anti-microbial resistance and phylogeny [15].

Diarrhea is one of the major causes of mortality in children under 5 years old in Iran, while the medical importance of diarrhea is reduced in older people in Iran. DEC is one the most important causative agents of diarrhea in children [6-9]. There is insufficient information on the frequency of DEC pathotypes in southwest Iran. The aims of this study were to determine the occurrence of DEC pathotypes isolated from children with diarrhea in the Khouzestan province, as well as the phylogenetic grouping of strains. DEC isolates were further characterized for their antimicrobial resistance profile, and ESBL production.

Methodology

Sampling and cultivation

During the period between September 2015 to June 2016, 208 stool samples from children under five years of age with diarrhea (characterized by three or more loose, liquid or watery stools or at least one bloody loose stool within 24 hours [16] were collected to determine the prevalence of diarrheagenic *E. coli* virulence genes. The study protocol conformed to the ethical guidelines of the Declaration of Helsinki (No 31400/2/3/95). The samples were collected from three main pediatric hospitals of Khouzestan province, Iran: Imam Khomeini, Golestan and Abouzar hospitals. Approximately 3 million people live in Khouzestan. The stool samples were cultured on MacConkey agar (Merck; Frankfurt, Germany) for the selection of *E. coli* isolates. All lactose- and non-lactose-fermenting colonies were tested due to EIEC strains which may be non-lactose fermenting. These colonies tested for diarrheagenic *E. coli* pathotypes markers by molecular methods. Conventional biochemical tests [17] were performed for all isolates and confirmed *E. coli* isolates were preserved at −30°C in brain heart infusion broth (Merck, Frankfurt, Germany) containing 20 % (v/v) glycerol for further analysis.

Bacterial lysates and multiplex-PCR

A multiplex-PCR assay was performed to detect the virulence genes of five categories of DEC in diarrheal stool samples. The virulence markers for determining DEC were defined as follows: for EPEC, the presence of *bfpB* and/or *escV* (strains containing *bfp* are typical EPEC and *escV* alone are atypical EPEC); for ETEC, the presence of *elt* and/or *estIa* or *estIb*; for STEC, the presence of *stx1* and/or *stx2* and possibly additional *escV*; for EIEC, the presence of *invE*; and for EAEC, the presence of *aggR* alone or with *astA* [17]. The *astA* was not specific for a certain pathogroup [17]; therefore, it was not considered in the final analysis.

One loop of a thickly growing bacterial colony from MacConkey agar plate was suspended in 500 µL water and also cultured on a MacConkey agar plate to achieve separate colonies. The mixture was boiled for 10 mins and then centrifuged for 10 mins at 11,337 *g* to pellet cell debris. The supernatant was used for the multiplex-PCR reaction [18]. The primers used are shown in Table 1. PCR conditions were performed as previously described by Muller *et al.* [17]. Briefly, multiplex-PCR was performed with the *Taq* DNA Polymerase 2× Master Mix Red (2 mM final $MgCl_2$ concentration) (Ampliqon, Odense, Denmark) using 1 µL of the supernatant as the template and PCR primers at the

concentrations listed in Table 1 in a 25 µL reaction volume. Thermo-cycling and time conditions were as follows: 94°C for 5 min, 30 cycles of 94°C for 30 s, 63°C for 30 s, and 72°C for 90 s with a final extension of 72°C for 5 minutes [17]. Six microliter of PCR product was then electrophoresed by 2% agarose gel. As reference strains, previously positive in-house control samples relative to each pathogroup confirmed by sequencing (microbial collection of Biology department, Shahid Chamran University of Ahvaz) and the *E. coli*-negative control (DH5a) were included for all runs.

Phylogenetic grouping

DNA was extracted from positive DEC isolates as described above and using triplex PCR described previously by Clermont *et al.* was subsequently subdivided into one of the four phylogenetic groups (A, B1, B2, and D). Two genes *chuA*, *yjaA*, and a DNA fragment TspE4.C2 were assayed by PCR reaction [19]. Sequences of used primers are showed in Table 1.

Screening for antimicrobial resistance and ESBL production

Antibiotic susceptibility to cefotaxime (30 µg), ceftazidime (30 µg), ceftriaxone (30 µg), ceftizoxime (30 µg), imipenem (10 µg), gentamicin (10 µg), ciprofloxacin (5 µg), piperacillin (100 µg), and amikacin (30 µg) was determined by the Kirby-Bauer disk diffusion method in accordance with the Clinical Laboratory Standard Institute (CLSI) guidelines [20]. Multi-drug resistance was defined as a resistance to three or more different antibiotic classes [21]. Identification of ESBL-producing isolates was performed by the synergy double disk (SDD) method using cefotaxime (30 µg) and ceftazidime (30 µg), alone and in combination with clavulanate (10 µg) with the distance between the discs was 20 mm center-to-center. As recommended by CLSI, an increase in diameter of the inhibition zone ≥ 5 mm when using the combined disc with clavulanate was considered as phenotypic evidence of ESBL production [20].

Table 1. Targets, names, sequences, and product sizes of primers used in this study.

Target gene or fragment	Primer	Sequence (5´to 3´)	Product size (bp)	Concentration (µM)	Reference
escV	MP3-escV-F	ATTCTGGCTCTCTTCTTCTTTATGGCTG	544	0.4	17
	MP3-escV-R	CGTCCCCTTTTACAAACTTCATCGC		0.4	
bfp	MP3-bfpB-F	GACACCTCATTGCTGAAGTCG	910	0.1	17
	MP3-bfpB-R	CCAGAACACCTCCGTTATGC		0.1	
*stx*1	MP4-stx1A-F	CGATGTTACGGTTTGTTACTGTGACAGC	244	0.2	17
	MP4-stx1A-R	AATGCCACGCTTCCCAGAATTG		0.2	
*stx*2	MP3-stx2A-F	GTTTTGACCATCTTCGTCTGATTATTGAG	324	0.4	17
	MP3-stx2A-R	AGCGTAAGGCTTCTGCTGTGAC		0.4	
elt	MP2-LT-F	GAACAGGAGGTTTCTGCGTTAGGTG	655	0.1	17
	MP2-LT-F	CTTTCAATGGCTTTTTTTTGGGAGTC		0.1	
estIa	MP4-STIa-F	CCTCTTTTAGYCAGACARCTGAATCASTTG	157	0.4	17
	MP4-STIa-R	CAGGCAGGATTACAACAAAGTTCACAG		0.4	
estIb	MP2-STI-F	TGTCTTTTTCACCTTTCGCTC	171	0.2	17
	MP2-STI-R	CGGTACAAGCAGGATTACAACAC		0.2	
invE	MP2-invE-F	CGATAGATGGCGAGAAATTATATCCCG	766	0.2	17
	MP2-invE-R	CGATCAAGAATCCCTAACAGAAGAATCAC		0.2	
astA	MP-astA-F	TGCCATCAACACAGTATATCCG	102	0.4	17
	MP-astA-R	ACGGCTTTGTAGTCCTTCCAT		0.4	
aggR	MP2-aggR-F	ACGCAGAGTTGCCTGATAAAG	400	0.2	17
	MP2-aggR-R	AATACAGAATCGTCAGCATCAGC		0.2	
chuA	chuA-1	GACGAACCAACGGTCAGGA	279	0.2	19
	chuA-2	TGCCGCCAGTACCAAAGACA		0.2	
yjaA	YjaA.1	TGAAGTGTCAGGAGACGCTG	211	0.2	19
	YjaA.2	ATGGAGAATGCGTTCCTCAAC		0.2	
TspE4C2	TspE4C2.1	GAGTAATGTCGGGGCATTCA	152	0.2	19
	TspE4C2.2	CGCGCCAACAAAGTATTACG		0.2	

Table 2. Frequency of diarrheagenic *E. coli* pathotypes obtained from children with diarrhea.

Pathotype	Genes	Number of isolates	N (%)
EAEC	*aggR*	14	35 (60.35)
	aggR+astA	21	
EPEC	*escV*	10	10 (17.25)
	bfpB	0	
ETEC	*estIa*	0	
	estIb	3	6 (10.34)
	elt	2	
	estIa+elt	1	
EIEC	*invE*	6	6 (10.34)
STEC	*stx1*	0	0 (0)
	stx2	0	
EAEC with LEE	*aggR+escV*	1	1 (1.72)

Statistical analysis

SPSS version 17 software was used for data analysis. A χ^2test or Fisher's exact test was performed to determine the statistical significance of the data. A P value of < 0.05 was considered significant.

Results

A total of 208 diarrheal samples from children less than five years old were tested for the presence of DEC. DEC was identified in 54 (26%) cases by multiplex PCR. As shown in Table 2, EAEC was the most frequent pathotypes isolated (60.35%), followed by EPEC (17.25%), EIEC (10.34%), and ETEC (10.34%). However, the STEC pathotype was not detected among DEC isolates. In total, 14 *E. coli* isolates harbored *astA* alone but due to association of the gene being found with multiple DEC pathotypes, these isolates were not considered as a distinct pathotype. Out of 54 positive samples, 4 (7%) cases contained more than one DEC pathotype. The combinations included two (3.5%) EAEC+EPEC, and two (3.5%) ETEC+EAEC, respectively. One *E. coli* isolate harbored two genes, *aggR* and *escV*, which was considered LEE-positive EAEC. The prevalence of each pathotypes virulence genes are shown in Table 2.

Antimicrobial resistance and ESBL producing

The results of antimicrobial resistance and ESBL-producing isolates of each pathotype is summarized in Table 3. The most prevalent resistance was ceftazidime (88%), followed by ceftizoxime (83%) and ceftriaxone (71%). Imipenem, ciprofloxacin, and amikacin were shown to be potentially effective antibiotics with a susceptible rate of 79%, 79%, and 78% respectively. Multi-drug resistance (resistance to ≥ 3 antimicrobial drug families) was demonstrated in 65.5% of DEC isolates with the highest frequency of MDR related to

Table 3. Phylogenetic analysis, antibiotic resistance patterns and ESBL production of diarrheagenic *E. coli* pathotypes.

	Phylogenetic group				Antibiotic resistance										
Pathotype N (%)	A	B1	B2	D	CRO	CP	AM	GM	FOX	CT	CAZ	CTX	IMP	ESBL Positive	MDR
	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)
EAEC35 (60.4)	6 (17.1)	6 (17.1)	15 (42.9)	8 (22.9)	23 (66)	7 (20)	9 (26)	13 (37)	13 (37)	29 (83)	30 (86)	22 (63)	5 (14)	26 (74)	21 (60)
EPEC10 (17.3)	3 (30)	1 (10)	6 (60)	0 (0)	8 (80)	4 (40)	2 (2)	2 (20)	4 (40)	9 (90)	9 (90)	7 (70)	4 (40)	9 (90)	8 (80)
EIEC6 (10.3)	3 (50)	1 (16.6)	1 (16.6)	1 (16.6)	4 (67)	0 (0)	1 (17)	3 (50)	1 (17)	4 (67)	5 (83)	5 (83)	0 (0)	5 (83)	4 (66.6)
ETEC6 (10.3)	3 (50)	0 (0)	3 (50)	0 (0)	5 (83)	1 (17)	1 (17)	0 (0)	2 (33)	5 (83)	6 (100)	5 (83)	3 (50)	6 (100)	4 (66.6)
EAEC with LEE 1 (1.7)	0 (0)	0 (0)	1 (3.9)	0 (0)	1 (100)	0 (0)	0 (0)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	0 (0)	0 (0)	1 (100)
Sum 58 (100)	15 (25.9)	8 (13.8)	26 (44.8)	9 (15.5)	41 (71)	12 (21)	13 (22)	19 (33)	21 (36)	48 (83)	51 (88)	40 (69)	12 (20.7)	46 (79.3)	38 (65.5)

CRO: ceftriaxone; CP: ciprofloxacin; AM: amikacin; GM: gentamicin; FOX: cefotaxime; CT: ceftizoxime; CAZ: ceftazidime; CTX: cefotaxime; IMP: imipenem; MDR: multi-drug resistant.

the EAEC pathotype. The overall incidence of ESBL-producing strains was 79.3% of all DEC isolates.

Phylogenetic analysis

As shown in Table 3, 44.8% isolates belonged to phylogenetic group B2, followed by phylogenetic group A, D, and B1 with frequency rates of 25.9%, 15.5%, and 13.8% respectively (Table 3). Phenotyping characterization of antimicrobial resistance of isolates according to phylogenetic groups is shown in Table 4. Patterns of antimicrobial resistance, ESBL-production, and MDR of DEC isolates were not significantly different among the four phylogenetic groups ($P > 0.05$).

Discussion

According to the method used in this study, it was revealed that 26% of children with diarrhea were infected with DEC. Our results are similar with previous reports regarding the prevalence of DEC infection in children less than 5 years old from Africa [22], Brazil [2], Iran [23], and Romania [24]. However, the reported frequencies of DEC are variable in other studies with reports showing frequencies of 9.4% in the United States [25], 14.1% in southeastern China [26], and 54% in Tehran, Iran [27].

In our study, more than 60% of DEC isolates carried virulence genes related to EAEC. The relatively high EAEC frequency (60.35%) was higher than that reported in other studies [22, 25-26]. The frequency of EAEC as 60% may not be due to sporadic infection but due to an undetermined outbreak; therefore, further typing would be required to determine if this high prevalence of EAEC is due to sporadic cases or an epidemic of an EAEC outbreak. However, this study is in agreement with other reports indicating a high prevalence of EAEC in diarrhea, suggesting EAEC is an important gastrointestinal pathogen [28]. Some *E. coli* isolates harbored only the *astA* gene, and due to some virulence genes such as *astA* which are widely distributed among the major *E. coli* pathotypes [24], these strains was not considered as independent pathotypes. Previous studies have reported that *astA* alone may not be associated with diarrhea [29,30]. The EAEC pathotype is not familiar to many healthcare workers; therefore, there is little information on the prevalence of this pathotype in subjects with or without diarrhea in Iran. EPEC was the second most prevalent isolated pathotype (17.25%) in DEC isolates and due to the *bfpB* gene not being detected in any of EPEC strains, these were considered as atypical-EPEC. The prevalence of ETEC and EIEC are indicates the potential of these pathotypes as pathogens responsible for diarrhea in children of the studied region of Khouzestan. The results indicated no prevalence of EHEC infection in this region and are in agreement with other studies performed in developing countries [4-5,31]. Different picture of DEC pathotype prevalence reveal gaps knowledge requiring further investigation.

One of the DEC isolates displayed marker virulence genes characteristic of two distinct pathotypes. In previous studies, these isolates were designated "intermediate strains" [17]. In this study, the detected isolate carried the EAEC regulatory gene *aggR* as well as the *escV* gene of EPEC. This profile was also reported by Liebchen *et al.* in Brazil [32]. This shows that DEC isolates may not harbor one particular virulence gene, but instead can harbor genes related to different pathotypes. Hereby, the bacteria may acquire additional virulence genes to enhance adaptation, and pathogenicity [32]. However, this study could not determine whether the gene combination occurred during infection or during isolation and passage. Unusual heterogeneity among EAEC strains has been well documented [33]. This event indicates the plasticity of the *E. coli* genome and highlights the need for surveillance on DEC infections [33]. In this study, three diarrheal samples showed mixed co-infections of

Table 4. Antibiotic resistance patterns, ESBL production, and MDR frequency in phylogenetic groups of diarrheagenic *E. coli* isolates.

Phylogenetic group (N, %)	CRO	CP	AM	GM	FOX	CT	CAZ	CTX	IMP	ESBL positive	MDR
	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)
A (15, 25.9)	11 (73.3)	2 (13.3)	3 (20)	4 (26.6)	5 (33.3)	12 (80)	12 (80)	11 (73.3)	5 (33.3)	12 (80)	10 (66.6)
B1 (8, 13.8)	7 (87.5)	4 (50)	3 (37.5)	2 (25)	2 (25)	8 (100)	8 (100)	7 (87.5)	2 (25)	6 (75)	5 (62.5)
B2 (26, 44.8)	16 (61.5)	4 (15.4)	6 (23.1)	9 (34.6)	12 (46.1)	20 (76.9)	23 (88.5)	18 (69.2)	4 (15.4)	21 (80.7)	16 (61.5)
D (9, 15.5)	7 (77.7)	2 (22.2)	1 (11.1)	4 (44.4)	2 (22.2)	8 (88.8)	8 (88.8)	4 (44.4)	1 (11.1)	6 (66.6)	7 (77.7)
Sum (58, 100)	41 (71)	12 (21)	13 (22)	19 (33)	21 (36)	48 (83)	51 (88)	40 (69)	12 (20.7)	46 (79.3)	38 (65.5)

CRO: ceftriaxone; CP: ciprofloxacin; AM: amikacin; GM: gentamicin; FOX: cefotaxime; CT: ceftizoxime; CAZ: ceftazidime; CTX: cefotaxime; IMP: imipenem; ESBL: Extended-spectrum beta-lactamase; MDR: multi-drug resistant.

two DEC pathotypes. The existence of mixed infections in diarrheal samples was also reported in other studies [5,34]. Based on the prevalence of different pathotypes in each region or country, it is recommended that causative agents of diarrhea be identified routinely in Iran. This information could be helpful to rapidly control infections and identify disease outbreaks.

Due to the changing pattern of antimicrobial resistance, understanding regional resistance patterns is essential for therapeutic decision making. According to Table 3 there was no significant relationship between DEC pathotype and rate of antibacterial resistance. One of the important findings in this study was the high rate of multi-drug resistance, observed in 65% of the DEC isolates. Resistance to these antimicrobial agents raises concerns about the limitation of pediatric therapeutic options. The increasing prevalence of multi-drug resistance in DEC strains is alarming. In our current study, the resistance rate to third generation cephalosporins (ceftriaxone, ceftazidime, cefotaxime and ceftizoxime) was higher than those reported in other studies [6,35-37]. Ceftriaxone and ceftazidime are widely-used antibiotics for the treatment of infectious diarrhea in children in Iran which may be a possible explanation for the high rate of DEC resistance to third generation cephalosporins. The common use of antimicrobials could contribute to higher selective pressure [24]. Some antibiotics such as ciprofloxacin are not used in children in Iran. Resistance to ciprofloxacin was similar to that reported in Shiraz, South Iran [38] but higher than in the United States (6%) [21], Spain (19.3%) [39], and Italy (5.3%)[39]. Ciprofloxacin resistance in this study may be due to transmission of resistant strains from adults, animals, or environments to children. Residues of antimicrobials in foods could also lead to the selection of resistant bacteria in the body [24]. Another important finding in this investigation was resistance (over 20%) of isolates to imipenem. Imipenem, a carbapenem antibiotic, has high activity against ESBL-producing *Enterobacteriaceae*. Generally, resistance to carbapenems is rare in clinical isolates and an increase in resistance should be properly monitored within a healthcare setting to inhibit an outbreak of resistant organisms. Gentamicin and amikacin from the aminoglycoside family were found to be effective against DEC isolates in this study; however, the observed resistance was higher than that reported by Aslani *et al.* [6] and Ghorbani-Dalini *et al.* [37]. Increases in antimicrobial resistance rates found in this study could be due to misuse and/or overuse of antibiotics leading to the transfer of resistance genes between bacteria and the spread of resistant isolates on a global scale.

Evaluating the pathogenic potential of each phylogenetic group showed a significant association between the B2 phylogenetic group and diarrheagenic isolates in comparison to the other phylogenetic groups. Almost half of the DEC isolates (44.8%) were assigned to the B2 phylogenetic group. Most of the isolates located in B2 phylogenetic group were related to the EAEC and EPEC pathotypes that have been reported by other researchers as phylogenetically heterogeneous [24]. In previous studies, analyzing DEC isolates in Costa Rica [40], Romania [24], Peru [13], and Iran [6] showed that most of the isolates belonged to B1, A, D, and B2 respectively. This reflects the diversity of DEC isolates in different countries.

Analyzing the relationship between phylogenetic group and antimicrobial resistance showed that there was no significant relationship, and all phylogenetic groups had a high rate of resistance and ESBL-producing isolates (Table 4). Although previous studies on extra intestinal strains reported that phylogenetic groups with more virulence factors, especially B2, are associated with lower levels of antimicrobial resistance [40] our finding showed a different result, in which the B2 and D phylogenetic groups exhibited high levels of antimicrobial resistance. This agrees with the findings reported by Mosquito *et al.* [13] showing the isolates belonging to high virulence phylogenetic groups also showed the highest levels of multidrug resistance.

Conclusion

Overall, our findings highlightthe importance of the role of DEC isolates in the etiology of diarrhea in children in Iran. EAEC was recovered at a high rate from diarrheal samples, indicating a wide spread of this pathotype in the studied population. To prevent outbreaks and reduce sporadic cases of diarrhea, its causative agents should be determined so that preventative measures can be taken. The progressive increase in antimicrobial resistance among DEC isolates makes it imperative to implement policies to control the spread of resistant bacteria.

Acknowledgements

The financial support of Shahid Chamran University of Ahvaz is acknowledged.

References

1. Garcia PG, Silva VL, Diniz CG (2011) Occurrence and antimicrobial drug susceptibility patterns of commensal and diarrheagenic *Escherichia coli* in fecal microbiota from

children with and without acute diarrhea. J Microbiol 49: 46-52.
2. Bueris V, Sircili MP, Taddei CR, dos Santos MF, Franzolin MR, Martinez MB, Ferrer SR, Barreto ML, Trabulsi LR. (2007) Detection of diarrheagenic *Escherichia coli* from children with and without diarrhea in Salvador, Bahia, Brazil. Mem Inst Oswaldo Cruz 2: 839-844.
3. Huang Z, Pan H, Zhang P, Cao X, Ju W, Wang C, Zhang J, Meng J, Yuan Z, Xu X (2016) Prevalence and antimicrobial resistance patterns of diarrheagenic *Escherichia coli* in Shanghai, China. Pediatr Infect Dis J 35: 835-859.
4. Ifeanyi CI, Ikeneche NF, Bassey BE, Al-Gallas N, Ben Aissa R, Boudabous A (2015) Diarrheagenic *Escherichia coli* pathotypes isolated from children with diarrhea in the Federal Capital Territory Abuja, Nigeria. J Infect Dev Ctries 9: 165-174. DOI: https://doi.org/10.3855/jidc.5528
5. Vilchez S, Reyes D, Paniagua M, Bucardo F, Möllby R, Weintraub A (2009) Prevalence of diarrhoeagenic *Escherichia coli* in children from León, Nicaragua. J Med Microbiol 58: 630-637.
6. Aslani MM, Salmanzadeh-Ahrabi S, Alikhani MY, Jafari F, Zali MR, Mani M (2008) Molecular detection and antimicrobial resistance of diarrheagenic *Escherichia coli* strains isolated from diarrheal cases. Saudi Med J 29: 388-392.
7. Alikhani MY, Hashemi SH, Aslani MM, Farajnia S (2013) Prevalence and antibiotic resistance patterns of diarrheagenic *Escherichia coli* isolated from adolescents and adults in Hamedan, Western Iran. Iran J Microbiol 5: 42-47.
8. Haghi F, Zeighami H, Hajiahmadi F, Khoshvaght H, Bayat M (2014) Frequency and antimicrobial resistance of diarrhoeagenic *Escherichia coli* from young children in Iran. J Med Microbiol 63: 427-432.
9. Salmanzadeh-Ahrabi S, Habibi E, Jaafari F, Zali MR. (2005) Molecular epidemiology of *Escherichia coli* diarrhoea in children in Tehran. Ann Trop Paediatr 25: 35-39.
10. Koochakzadeh A, Askari Badouei M, Mazandarani E, Madadgar O (2014) Survey on O157:H7 enterohemorrhagic *Escherichia coli* (EHEC) in cattle in Golestan province, Iran. Iran J Microbiol 6: 276-280.
11. Paterson DL, Bonomo RA (2005) Extended-spectrum β-Lactamases: a clinical update. Clin Microbiol Rev 18: 657-686.
12. Salmani H, Azarnezhad A, Fayazi MR, Hosseini A (2016) Pathotypic and phylogenetic study of diarrheagenic *Escherichia coli* and uropathogenic *E. coli* using multiplex Polymerase Chain Reaction. Jundishapur J Microbiol 9: e28331.
13. Mosquito S, Pons MJ, Riveros M, Ruiz J, Ochoa TJ (2015) Diarrheagenic *Escherichia coli* phylogroups are associated with antibiotic resistance and duration of diarrheal episode. Sci World J 2015: 610403.
14. Ahmed OB, Omar AO, Asghar AH, Elhassan MM (2013) Prevalence of TEM, SHV and CTX-M genes in *Escherichia coli* and *Klebsiella* spp urinary isolates from Sudan with confirmed ESBL phenotype. Life Sci J 10: 191-195.
15. Hannah EL, Johnson JR, Angulo F, Haddadin B, Williamson J, Samore MH (2009) Molecular analysis of antimicrobial-susceptible and -resistant *Escherichia coli* from retail meats and human stool and clinical specimens in a rural community setting. Food borne Pathog Dis 6: 285–295.
16. Mason E (2006) WHO's strategy on integrated management of childhood illness. Bull World Health Organ 84: 594-596.
17. Müller D, Greune L, Heusipp G, Karch H, Fruth A, Tschäpe H, Schmidt MA (2007) Identification of unconventional intestinal pathogenic *Escherichia coli* isolates expressing intermediate virulence factor profiles by using a novel single-step multiplex PCR. Appl Environ Microbiol 73: 3380-3390.
18. Bonkoungou IJ, Lienemann T, Martikainen O, Dembelé R, Sanou I, Traoré AS, Siitonen A, Barro N, Haukka K (2012) Diarrhoeagenic *Escherichia coli* detected by 16-plex PCR in children with and without diarrhoea in Burkina Faso. Clin Microbiol Infect 18: 901-906.
19. Clermont O, Bonacorsi S, Bingen E (2000) Rapid and simple determination of the *Escherichia coli* phylogenetic group. Appl Environ Microbiol 66: 4555-4558.
20. Clinical and Laboratory standard institute (CLSI) (2015) Performance Standards for Antimicrobial Susceptibility Testing; 25th informational supplement document. M100 –S25 (ISBN 1-56238-989-0).
21. Diekema DJ, Boots Miller BJ, Vaughn TE, Woolson RF, Yankey JW, Ernst EJ, Flach SD, Ward MM, Franciscus CL, Pfaller MA, Doebbeling BN (2004) Antimicrobial resistance trends and outbreak frequency in United States hospitals. Clin Infect Dis 38: 78-85.
22. Moyo SJ, Maselle SY, Matee MI, Langeland N, Mylvaganam H (2007) Identification of diarrheagenic *Escherichia coli* isolated from infants and children in Dar es Salaam, Tanzania. BMC Infect Dis 7: 92.
23. Heidary M, Momtaz H, Madani M (2014) Characterization of diarrheagenic antimicrobial resistant *Escherichia coli* isolated from pediatric patients inTehran, Iran. Iran Red Crescent Med J 16: e12329.
24. Usein CR, Tatu-Chitoiu D, Ciontea S, Condei M, Damian M (2009) *Escherichia coli* pathotypes associated with diarrhea in Romanian children younger than 5 years of age. Jpn J Infect Dis 62: 289-293.
25. Nataro JP, Mai V, Johnson J, Blackwelder WC, Heimer R, Tirrell S, Edberg SC, Braden CR, Glenn Morris J Jr, Hirshon JM (2006) Diarrheagenic *Escherichia coli* infection in Baltimore, Maryland, and New Haven Connecticut. Clin Infect Dis 43: 402–407.
26. Chen Y, Chen X, Zheng S, Yu F, Kong H, Yang Q, Cui D, Chen N, Lou B, Li X, Tian L, Yang X, Xie G, Dong Y, Qin Z, Han D, Wang Y, Zhang W, Tang YW, Li L (2014) Serotypes, genotypes and antimicrobial resistance patterns of human diarrhoeagenic *Escherichia coli* isolates circulating in southeastern China. Clin Microbiol Infect 20: 52-58.
27. Jafari F, Hamidian M, Rezadehbashi M, Doyle M, Salmanzadeh-Ahrabi S, Derakhshan F, Reza Zali M (2009) Prevalence and antimicrobial resistance of diarrheagenic *Escherichia coli* and *Shigella* species associated with acute diarrhea in Tehran, Iran. Can J Infect Dis Med Microbiol 20: 56–62.
28. Wilson A, Evans J, Chart H, Cheasty T, Wheeler JG, Tompkins D, Smith HR (2001) Characterisation of strains of enteroaggregative *Escherichia coli* isolated during the infectious intestinal disease study in England. Eur J Epidemiol 17: 1125-1130.
29. Rajendran P, Ajjampur SS, Chidambaram D, Chandrabose G, Thangaraj B, Sarkar R, Samuel P, Rajan DP, Kang G (2010) Pathotypes of diarrheagenic *Escherichia coli* in children attending a tertiary care hospital in South India. Diagn Microbiol Infect Dis 68: 117-122.
30. Jenkins C, Tembo M, Chart H, Cheasty T, Willshaw GA, Phillips AD, Tompkins D, Smith H (2006) Detection of enteroaggregative *Escherichia coli* in faecal samples from

patients in the community with diarrhoea. J Med Microbiol 55: 1493–1497.
31. Hegde A, Ballal M, Shenoy S (2012) Detection of diarrheagenic *Escherichia coli* by multiplex PCR. Indian J of Medical Microbiol 30: 279-284.
32. Liebchen A, Benz I, Mellmann A, Karch H, Gomes TA, Yamamoto D, Hernandes RT, Sampaio J, Sampaio SC, Fruth A, Schmidt MA (2011) Characterization of *Escherichia coli* strains isolated from patients with diarrhea in Sao Paulo, Brazil: identification of intermediate virulence factor profiles by multiplex PCR. J Clin Microbiol 49: 2274-2278.
33. Jafari A, Aslani MM, Bouzari S (2012) *Escherichia coli*: a brief review of diarrheagenic pathotypes and their role in diarrheal diseases in Iran. Iran J Microbiol 4: 102-117.
34. Abba K, Sinfield R, Hart CA, Garner P (2009) Pathogens associated with persistent diarrhea in children in low and middle income countries: systematic review. BMC Infect Dis 9: 88.
35. Zeighami H, Haghi F, Hajiahmadi F, Kashefiyeh M, Memariani M. (2015) Multi-drug-resistant enterotoxigenic and enterohemorrhagic *Escherichia coli* isolated from children with diarrhea. J Chemother 27: 152-125.
36. Garcia PG, Silva VL, Diniz CG (2011) Occurrence and antimicrobial drug susceptibility patterns of commensal and diarrheagenic *Escherichia coli* in fecal microbiota from children with and without acute diarrhea. J Microbiol 49: 46-52.
37. Ghorbani-Dalini S, Kargar M, Doosti A, Abbasi P, Sarshar M (2015) Molecular epidemiology of ESBL genes and multi-drug resistance in diarrheagenic *Escherichia coli* strains isolated from adults in Iran. Iran J Pharm Res 14: 1257-1262.
38. Rezaee MA, Sheikhalizadeh V, Hasani A (2011) Detection of integrons among multi-drug resistant (MDR) *Escherichia coli* strains isolated from clinical specimens in northern west of Iran. Braz J Microbiol 42: 1308-1313.
39. Oteo J, Lazaro E, de Abajo FJ, Baquero F, Campos J (2005) Antimicrobial-resistant invasive *Escherichia coli*, Spain. Emerg Infect Dis 11: 546-553.
40. Pérez C, Gómez-Duarte OG, Arias ML (2010) Diarrheagenic *Escherichia coli* in children from Costa Rica. Am J Trop Med Hyg 83: 292-297.

Corresponding author

Seyedeh Elham Rezatofighi, PhD
Department of Biology, Faculty of Science, Shahid Chamran University of Ahvaz, Golestan Blv, Postal code:6135743135, Ahvaz, Iran.
Phone and Fax: 00986133331045
Email: e.tofighi@scu.ac.ir

Conflict of interests: No conflict of interests is declared.