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Characterization of Antimicrobial Susceptibility, Extended-Spectrum β-Lactamase Genes and Phylogenetic Groups of Enteropathogenic *Escherichia coli* Isolated from Patients with Diarrhea

Erfaneh Jafari, Saeid Mostaan, Saeid Bouzari *

Molecular Biology Department, Pasteur Institute of Iran, Tehran, Iran

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ABSTRACT

Objectives: Infectious diarrhea is one of the most common causes of pediatric death worldwide and enteropathogenic *Escherichia coli* (EPEC) is one of the main causes. There are 2 subgroups of EPEC, typical and atypical, based on the presence or absence of bundle forming pili (bfp), of which atypical EPEC is considered less virulent, but not less pathogenic. Antimicrobial resistance towards atypical EPEC among children is growing and is considered a major problem. In this study the pattern of antibiotic resistance in clinical isolates was determined.

Methods: Using 130 isolates, antibiotic resistance patterns and phenotypes were assessed, and genotypic profiles of extended spectrum β-lactamase (ESBL) production using disc diffusion and PCR was carried out. Phylogenetic groups were analyzed using quadruplex PCR.

Results: There were 65 *E. coli* isolates identified as atypical EPEC by PCR, among which the highest antibiotic resistance was towards ampicillin, followed by trimethoprim-sulfamethoxazole, and tetracycline. Multidrug resistance was detected in 44.6% of atypical EPEC isolates. Around 33% of isolates were determined to be extended spectrum β-lactamase producers, and in 90% of isolates, genes responsible for ESBL production could be detected. Moreover, the majority of atypical EPEC strains belonged to Group E, followed by Groups B1, B2 and C.

Conclusion: High rates of multidrug resistance and ESBL production among atypical EPEC isolates warrant periodical surveillance studies to select effective antibiotic treatment for patients. It is considered a critical step to manage antibiotic resistance by avoiding unnecessary prescriptions for antibiotics.

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Introduction

Escherichia coli (E. Coli) in general are a common human pathogen with 6 known pathotypes, among which enteropathogenic *E. coli* (EPEC) causes the majority of cases of diarrhea in developing countries, and is considered the main cause of infant mortality [1]. Based on the present or absence of bundle forming pili (bfp), 2 subgroups of EPEC (typical EPEC and atypical EPEC) have been identified [1]. Due to the absence of the *E. coli* adherence factor EAF plasmid which encodes bfp, atypical EPEC are considered less virulent, although they are not less pathogenic [2], and have been emerging more in recent years [3]. Atypical EPEC pathogenesis involves an attaching and an effacing (A/E) lesion, and the genes that are responsible for the production of these lesions are called intimin (*eae*) and translocated receptor (*tir*). These

*Corresponding author: Saeid Bouzari
Molecular Biology Department, Pasteur Institute of Iran, Tehran, Iran
E-mail: saeidbouzari@yahoo.com
ORCID: Erfaneh Jafari https://orcid.org/ 0000-0002-1122-4523, Saeid Mostaan https://orcid.org/0000-0003-1892-0019, Saeid Bouzari https://orcid.org/ 0000-0002-3134-9665

genes are located on a pathogenicity island named the Locus of Enterocyte Effacement. The interaction of *eae* and *tir* are responsible for the attachment of bacteria to host cells, and actin pedestal formation in intestinal cells which characterizes the A/E pathogens [1].

Extended spectrum β-lactamases (ESBLs) are specific enzymes which are bacterial chromosome mediated and plasmid mediated [4]. ESBLs may include Class A, C or D enzymes that are generally inhibited by clavulanic acid, and their most common spread mechanism is by horizontal gene transfer [5]. Class A enzymes including TEM, SHV, and CTX-M, and Class D enzyme OXA are mostly found among *Enterobacteriaceae* [5]. As ESBL producing organisms also display resistance to other classes of antibiotics [6], they lead to the development of multidrug resistant (MDR) strains. In recent years, due to the excess prescription of various antibiotics (such as β-lactams) against infections, high levels of antibiotic resistance are being detected which are linked to both typical and atypical EPEC strains [7,8]. Therefore, the selection of effective antibiotics for patients is considered critical [9,10], together with reduced prescriptions for antibiotics.

Phylogenetic analysis of *E.coli* clinical strains provides information about the frequency of occurrence in the environment, and based on the presence or absence of certain genes and DNA fragments (including *chuA, yjaA, arpA* genes and a DNA fragment TspE4.C2), *E.coli* can be classified into phylogenetic groups (A, B1, B2, C, D, E and F) [11]. Majority of commensal *E.coli* strains belong to phylogroups A and B1 [12], and extraintestinal pathogenic *E.coli* mainly belongs to phylogroups B2 and D [13]. However, as the arrangement of phylogroups for diarrheagenic *E. coli* is still unclear, EPEC could belong to any of the phylogroups [14,15].

In the present study, antibacterial resistance patterns, ESBL production and phylogenetic groups associated with clinical isolates were evaluated.

Materials and Methods

1. Sampling and processing

Stool samples from patients with diarrhea inoculated on MacConkey agar were collected from different reference hospitals during a 1-year period 130 samples from varying age groups (58 patients under 6 years old, 34 patients between 10-30 years, and 38 patients older than 30 years) had been biochemically confirmed as *E. coli* and 5 colonies from each of their MacConkey agar plate tested by PCR to identify the presence of *eae* (F primer, AGGCTTCGTCACAGTTG and R primer, CCATCGTCACCAGAGGA) [16] or *bfp* (F primer, GACACCTCATTGCTGAAGTCG and R primer, CCAGAACACCTCCGTTATGC) [17], as EPEC virulence genes and absence of *stx1* (F primer, CGATGTTACGGTTTGTTACTGTGACAGC and R primer, AATGCCACGCTTCCCAGAATTG) and *stx2* (F primer, GTTTTGACCATCTTCGTCTGATTATTGAG and R primer, AGCGTAAGGCTTCTGCTGTGAC) genes [17]. *E. coli* strain E2348/69 was used as a positive control for both *eae* and *bfp* genes, and *E. coli* strain O157/H7 was used as a positive control for *stx1* and *stx2* genes. Non-pathogenic *E. coli* strain DH5α was used as negative control to monitor PCR contamination. PCR conditions for the detection of virulence genes were as follows: 95°C for 3 minutes, 30 cycles of 94°C for 1 minute, 60°C for 45 seconds, 72°C for 45 seconds, and a final extension at 72°C for 5 minutes.

All sampling protocols in this study were approved by the ethics committee of the Pasteur Institute, Iran (ethical no.: IR.PII.REC.1397.003).

2. Antimicrobial susceptibility testing

Antimicrobial susceptibility tests were performed on Mueller-Hinton agar (Himedia, India) using commercial antimicrobial discs (BD BBL, USA), based on Kirby-Bauer's method [18] according to the guidelines of the clinical and laboratory standards institute [19].

The antibiotics used were ampicillin (10 μg), amikacin (30 μg), ceftazidime (30 μg), cefotaxime (30 μg), ciprofloxacin (5 μg), ertapenem (10 μg), imipenem (10 μg), levofloxacin (5 μg), piperacillin-tazobactam (100 + 10 μg), tetracycline (30 μg) and trimethoprim-sulfamethoxazole (1.25 + 23.75 μg).

Multidrug resistance was defined by discerning non-susceptibility to at least 1 antibiotic in 3 or more antimicrobial categories [20]. *E. coli* ATCC 25922 was used for quality control.

3. Phenotypic characterization of ESBL producing strains

Detection of ESBLs were performed using the double disk synergy test method [19] using ceftazidime (30 μg) and cefotaxime (30 μg) discs (BD BBL,USA), and placing each from a disc containing ceftazidime + clavulanic acid (30 + 10 μg) and cefotaxime + clavulanic acid (30 + 10 μg) (BD BBL, USA), respectively. ESBL producing was determined by the expansion of ≥ 5 mm of the zone diameters of combined discs compared to ceftazidime and cefotaxime zones.

4. Genotypic characterization of ESBL producing strains

β-lactamase genes *blaCTX-M9* (F primer, GTGACAAAGAGA GTGCAACGG and R primer, ATGATTCTCGCCGCTGAAGCC) [21], *blaSHV* (F primer, TCGCCTGTGTATTATCTCCC and R primer, CGCAGATAAATCACCACAATG) [22], *blaOXA* (F primer, GCGTGGTTAAGGATGAACAC and R primer, CATCAAGTTCAACCCAACCG) [23] and *blaTEM* (F primer, GCGGAACCCCTATTTG and R primer, ACCAATGCTTAATCA GTGAG) [24] were detected by PCR using these specific primers.

DNA was extracted by boiling method and the PCR procedure was performed in a total volume of 25 µL, using 12 µL of Taq DNA Polymerase Mix Red-$Mgcl_2$ 2 mM (Ampliqon), 9 µL of DNase/RNase free distilled water (ThermoScientific), 1 µL of 10 pM for reverse and forward primers, and 2 µL of DNA template.

5. Identification of phylogroups

All the EPEC strains were assessed for phylogenetic groups A, B1, B2, C, D, E and F using quadruplex multiplex PCR [11]. Agarose gel electrophoresis of the PCR product was carried out in 2% agarose gel containing DNA Gel dye.

6. Statistical analysis

Pearson correlation test was performed using SPSS Version 18 (Spss Inc., Chicago, IL, USA) for statistical analysis of the data. Correlation is significant at the 0.01 level (2-tailed).

Results

There were a total of 130 *E.coli* isolates tested for *eae*, *bfp*, *stx1,* and *stx 2* genes using PCR; from which 65 isolates (50%) were positive for the *eae* gene and negative for *bfp, stx1,* and *stx2* genes. None of the isolates were positive for the *bfp* gene, the characteristic of typical EPEC, therefore they were classified as atypical EPEC. Of the positive isolates, 30 (46.15%) belonged to children under 6 years, 21 (32.3%) belonged to 10-30 year olds, and the rest of the isolates were from patients older than 30 years.

The antibiotic susceptibility test (Tables 1 and 2), showed that among the 65 atypical EPEC isolates positive for the *eae* gene, 21.5% were sensitive to all 11 antibiotic discs, and 78.5% showed resistance to at least 1 of the antibiotic discs. From these isolates 33.8% (22 of 65) were ESBL producers.

The highest resistance to an antibiotic was towards ampicillin (70.8%), followed by trimethoprim-sulfamethoxazole (56.9%), and tetracycline (46.2%). All isolates were susceptible to imipenem and ertapenem. Most of the isolates were susceptible to piperacillin-tazobactam, and amikacin, except for 2 isolates that showed resistance to piperacillin-tazobactam. All ESBL positive isolates were also susceptible to imipenem, ertapenem, piperacillin-tazobactam and amikacin, except for 1 isolate that showed resistance to piperacillin-tazobactam. Multidrug resistance was detected in 29 (44.6%) atypical EPEC isolates, and 58.62% of MDR isolates were ESBL producing. In addition, 51.7% of children under 6 had isolates which displayed MDR.

There was a strong, positive correlation between ESBL positive and cefotaxime resistance, which was statistically significant (r = 0.914, n = 65, p < 0.001). There were also moderate, significant positive correlation between ESBL positive and ceftazidime, ciprofloxacin, or trimethoprim-sulfa resistance (r = 0. 598, 0.329, 0.367, respectively, n = 65, p < 0.01). There was a weak, significant positive correlation between ESBL positive and levofloxacin resistance (r = 0.282, n = 65, p = 0.023). Finally, there was no correlation between ESBL positive and piperacillin-tazo, ertapenem, imipenem, tetracyclines, amikacin and ampicillin resistance (Table 2).

Table 1. Antimicrobial susceptibility patterns.

Antibiotic	All atypical EPEC isolates (n = 65)		
	Resistant	Intermediate	Sensitive
Cefotaxime	23 (35.4)	1 (1.53)	41 (63.1)
Ceftazidime	11 (16.9)	4 (6.15)	50 (76.9)
Piperacillin-tazo	2 (3.1)	1 (1.53)	62 (95.4)
Ertapenem	-	-	65 (100)
Imipenem	-	-	65 (100)
Ciprofloxacin	5 (7.7)	8 (12.3)	52 (80)
Tetracyclines	30 (46.2)	3 (4.61)	32 (49.2)
Amikacin	-	3 (4.61)	62 (95.4)
Levofloxacin	5 (7.7)	-	60 (92.3)
Ampicillin	46 (70.8)	4 (6.15)	15 (23.1)
Trimethoprim-sulfa	37 (56.9)	2 (3.07)	26 (40)

Data are presented as n (%).
EPEC = *escherichia coli.*

Studying 4, β-lactamase producing genes in 22 ESBL positive isolates (using PCR) showed 20 (90.9%) isolates were positive at least for 1 of these ESBL encoding genes. The *blaCTX-M9* gene was detected in 15 strains (68.2%), *blaSHV* gene in 10 strains (45.4%), *blaOXA* gene in 9 strains (40.9%) and *blaTEM* gene in 12 strains (54.5%). According to molecular resistance profile of ESBL-producing isolates, 2 strains contained all 4 genes, 3 and 2 genes patterns were detected in 6, and 8 strains, and 4 strains had only 1 of the evaluated genes (Table 3).

Furthermore, comparison of phenotypic and genotypic antimicrobial patterns in ESBL producing isolates showed all strains containing ESBL encoding genes were also susceptible to amikacin. *CTX-M9* positive strains had a high rate of resistance to trimethoprim-sulfamethoxazole (73.3%) and tetracycline (33.3%). *TEM* and *OXA* positive strains also had a high rate of resistance to trimethoprim-sulfamethoxazole (91.7%), (100%) and tetracycline (66.7%), (44.4%), respectively; and *SHV* positive strains had a high rate of resistance to trimethoprim-sulfamethoxazole (70%).

In Table 4, phylogenic grouping of the EPEC isolates were

Table 2. Antimicrobial susceptibility patterns.

Antibiotic	ESBL– isolates (*n* = 43)			ESBL+ isolates (*n* = 22)			*p*
	Resistant	Intermediate	Sensitive	Resistant	Intermediate	Sensitive	
Cefotaxime	2 (4.6)	-	41 (95.3)	21 (95.5)	1 (4.5)	-	< 0.001
Ceftazidime	2 (4.6)	-	41 (95.3)	9 (40.9)	4 (18.2)	9 (40.9)	< 0.001
Piperacillin-tazo	1 (2.3)	1 (2.3)	41 (95.3)	1 (4.5)	-	21 (95.5)	NS
Ertapenem	-	-	43 (100)	-	-	22 (100)	-
Imipenem	-	-	43 (100)	-	-	22 (100)	-
Ciprofloxacin	1 (2.3)	3 (7.0)		4 (18.2)	5 (22.7)		0.009
Tetracyclines	21 (48.8)	1 (2.3)		9 (40.9)	2 (9.1)		NS
Amikacin	-	3 (7.0)		-	-		NS
Levofloxacin	1 (2.3)	-		4 (18.2)	-		0.023
Ampicillin	24 (55.8)	4 (9.3)		22 (100)	-		0.001
Trimethoprim-sulfa	19 (44.2)	1 (2.3)		18 (81.8)	1 (4.5)		0.008

Data are presented as *n* (%).
ESBL = extended spectrum β-lactamase; NS = not significant.

Table 3. Genotypic pattern of 22 ESBL positive strains.

Resistance pattern	*n* (%)
CTX-M9/SHV/OXA/TEM	2 (9.1)
CTX-M9/SHV/OXA	1 (4.5)
CTX-M9/SHV/TEM	2 (9.1)
CTX-M9/OXA/TEM	3 (13.6)
CTX-M9/SHV	3 (13.6)
CTX-M9/OXA	1 (4.5)
CTX-M9/TEM	2 (9.1)
SHV/TEM	2 (9.1)
CTX-M9	1 (4.5)
OXA	2 (9.1)
TEM	1 (4.5)
None	2 (9.1)

ESBL = extended spectrum β-lactamase.

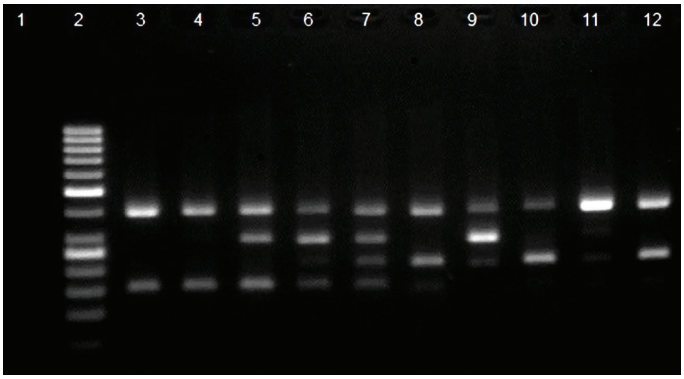

Figure 1. Phylogrouping of EPEC strains on 2% agarose gel. *arpA* (400bp), *chuA* (288bp), *yjaA* (211bp) and TspE4.C2 (152bp). Lane 1, negative control; lane 2, molecular weight marker (50bp, Fermentas); lanes 3&4, group B1 (+ − − +); lanes 5&8, group E (+ + − +); lane 6&7, unknown (+ + + +); lanes 9&11, group E (+ + + −) and lanes 10&12, group C (+ − + −).

assigned according to the Clermont's quadruplex phylogroup method (Figure 1). These results revealed Group E as the predominant phylogroup (26.2% occurrence), followed by Groups B1 (20%), B2 (13.9%) and C (12.3%). Groups A and D showed a prevalence of 3.07%; moreover approximately 18.5% of the isolates remained unclassified, and no strains belonged to Group F.

In addition, the antimicrobial resistance pattern among atypical EPEC phylogroups showed that a greater number of resistance patterns were seen in group E, followed by Groups B1, C and B2. Furthermore, 50% of ESBL positive isolates belonged to Group E (Table 5).

Table 4. Phylogenetic grouping.

Phylogenetic group	All atypical EPEC isolates (*n* = 65)	ESBL– isolates (*n* = 43)	ESBL+ isolates (*n* = 22)
	n (%)	*n* (%)	*n* (%)
A	2 (3.07)	1 (2.3)	1 (4.5)
B1	13 (20)	13 (30.2)	-
B2	9 (13.84)	6 (13.95)	3 (13.6)
C	8 (12.3)	5 (11.6)	3 (13.6)
D	2 (3.07)	-	2 (9.1)
E	17 (26.15)	6 (13.95)	11 (50)
F	-	-	-
Clade I	2 (3.07)	2 (4.65)	-
Unknown	12 (18.5)	10 (23.25)	2 (9.1)

EPEC = *escherichia coli*; ESBL = extended spectrum β-lactamase.

Table 5. Antibiotic resistance patterns in atypical EPEC phylogenetic groups.

Phylogenetic group	CTX	CAZ	TZP	ETP	IPM	CIP	Te	AN	LVX	AM	SXT
	No.	No.	No.	No.	No.	No.	No.	No.	No.	No.	No.
A (*n* = 2)	2	2	1	0	0	1	2	0	1	2	2
B1 (*n* = 13)	1	0	0	0	0	1	8	0	1	9	7
B2 (*n* = 9)	3	2	0	0	0	0	4	0	0	6	4
C (*n* = 8)	3	0	0	0	0	0	3	0	0	7	4
D (*n* = 2)	2	1	0	0	0	0	0	0	0	2	0
E (*n* = 17)	10	5	0	0	0	3	7	0	3	14	13
Clade I (*n* = 2)	0	0	0	0	0	0	1	0	0	1	1
Unknown (*n* = 12)	2	1	1	0	0	0	5	0	0	5	6

AM = ampicillin; AN = amikacin; CAZ = ceftazidime; CIP = ciprofloxacin; CTX = cefotaxime; EPEC = *escherichia coli*; ETP = ertapenem; IPM = imipenem; LVX = levofloxacin; STX = trimethoprim + sulfamethoxazole; Te = tetracyclines; TZP = piperacillin + tazobactam.

Discussion

EPEC are among the most predominant *E. coli* pathotypes in children under 5 [25,26], and one of the most important fatal pediatric pathogens in the developing world which is responsible for nearly 1.6-2.5 million infant deaths each year [9,25,27].

While assessing virulence genes among *E. coli* isolates, only atypical EPEC subtypes were identified, which is in accordance with an increased incidence of atypical EPEC in different studies [28-33]. Antibacterial assessment of 11 antibiotics in 65 atypical EPEC isolates demonstrated high rates of antibacterial resistance. These results are in agreement with other findings [3,28]. Over prescription and overuse of antibiotics is the main cause of resistance emergence among isolates, which is a major public health concern.

In the current study, high levels of resistance to ampicillin, trimethoprim-sulfamethoxazole and tetracycline were observed, which is in agreement with previous findings in India, China, Iran, Brazil, Tanzania and Peru [2,3,28,34-36]. Compared with the studies by Bakhshi et al [33] and Memariani et al [37], our study revealed lower rates of ciprofloxacin resistance in atypical EPEC isolates.

The incidence of multidrug resistance among the isolates in this current study was 44.6%, which was lower than other studies conducted in Iran [28,38], while sensitivity toward

imipenem and ertapenem, was similar to some studies [38,39]. The majority of the isolates were also susceptible to amikacin and piperacillin-tazobactam showing their effectiveness against atypical EPEC clinical strains.

ESBL production was observed in 33.8% of strains however, 77.3% of these strains showed MDR. There was 95.5% resistance to cefotaxime which was higher than a prior report [37]. The genotypic method, in this study showed 90.9% of the ESBL positive strains carried at least 1 ESBL encoding gene, among which *CTX-M9* was the most common (68.2%), followed by *TEM* (54.5%), *SHV* (45.4%), and *OXA* (40.9%). These results are different from the previous study performed by Singh et al [2], although the prevalence of the *SHV* gene was higher than those reported in other studies [3,38].

The results in this current study showed a significant statistical difference in cefotaxime, ceftazidime, ciprofloxacin, levofloxacin, ampicillin and trimethoprim-sulfamethoxazole resistance between ESBL positive and ESBL negative isolates, which is in agreement with another study [40], and supports the fact that ESBL encoding plasmids also carry resistance genes for other antimicrobial drugs.

Phylogenetic evaluations revealed that the majority of atypical EPEC isolates belonged to 1 of 4 phylogroups; E, B1, B2 and C, and represented more affiliation with Group E, which was different to the findings from other studies [2,29,38,41]. None of the isolates were associated with Group F, which is in accordance with other reported studies [2,41].

Observing the relationship between phylogenetic group and antimicrobial pattern among EPEC phylogroups, the results showed that all of the B1 groups, and the majority of B2 groups, belonged to non-ESBL producing atypical EPEC, whereas half of the ESBL positive isolates, as well as the majority of MDR isolates belonged to Group E. High rates of resistance in phylogroup E was due to ampicillin and trimethoprim-sulfamethoxazole, followed by cefotaxime and tetracycline resistance. Whereas resistance of Groups B1 and B2 was due to ampicillin, tetracycline, and trimethoprim-sulfamethoxazole resistance. Group E also presented a higher percentage of MDR than other groups.

In conclusion, this study indicated resistance emergence among isolates, which is a major public health concern. Therefore, periodical surveillance studies to select effective antibiotics for patients, is considered a critical step to manage *E. coli* resistance, in addition to overprescribing antibiotics to patients.

Conflicts of Interest

The authors report there were no conflicts of interest in this work.

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