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Original article

Antibacterial resistance patterns of extended spectrum β-lactamase - producing enteropathogenic *Escherichia coli* strains isolated from children

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ABSTRACT

Background and study aim: This study aimed to determine the antibacterial resistance patterns of extended spectrum β-lactamase (ESBL)-producing enteropathogenic *Escherichia coli* (EPEC) isolated from Iranian children and to investigate its genetic patterns.

Patients and methods: 192 non-repeats EPEC isolates were collected from stool samples of the children with and without diarrhoea. The EPEC strains were isolated from 1355 stool specimens obtained from 247 children with diarrhoea (0–10 years old; mean age, 5.5 years) and 1108 children without any gastrointestinal symptoms (0–10 years old; mean age, 6.8 years) during the summer months in three Iranian provinces, Tehran, Ilam and Mazandaran. Strains biochemically identified as *E. coli* were selected and were identified by the presence of *eaeA* and *bfpA* as EPEC virulence genes. Antimicrobial susceptibilities were determined by disc diffusion method. The isolates were confirmed to be ESBL producers by the double disk synergy test (DDST). The β-lactamase genes (*blaTEM, blaSHV, blaCTX-M, blaOXA*) and insertion sequence *ISEcp1* were detected by PCR method.

Results: The highest antibiotic susceptibility was detected to imipenem (100%), followed by gentamicin (82.3%) and ciprofloxacin (79.2%). The highest resistance was detected to cefpodoxime (97.9%), trimethoprim (60.7%), and tetracycline (58.4%), respectively. Totally, 153 EPEC strains (79.7%) were ESBL-producing by DDST test. The PCR showed that 84 (43.8%) EPEC isolates were positive for ESBLs encoding genes. Among 153 ESBLs-producing EPEC, *TEM* was present in 9.2% of isolates. Also, *CTX-M* and *SHV* genes were detected in 7.2% and 7.8%, respectively. The *SHV* positive strains were associated with the highest resistance rate to tetracycline (56.5%), although the TEM and OXA were associated with the highest resistance rate to gentamicin (23.1%) and ciprofloxacin (21.4%).

Conclusions: The study revealed that 79.7% of EPEC isolates from Iranian children were ESBL-producing and were comparable with the non ESBL-producing isolates regarding susceptibility to the antibiotics.

Introduction

Enterobacteriaceae are rod-shaped Gram-negative bacteria, many members of which are normal part of the flora. *Escherichia coli*, a member of the Enterobacteriaceae are considered one of the most common human pathogens [1]. Enteropathogenic *Escherichia coli* is a major cause of diarrhoea in developing countries [2]. The increase in *Escherichia coli* resistance to various antibacterial agents is now a major concern. The resistance to antibiotics in different populations around the world is very high [3,4]. Due to the indiscriminate use of various antibiotics against this pathogen, resistant strains increased in recent years. In addition, the development of multi-drug resistant strains causes problems in the treatment of infections caused by *E. coli*, especially intra and extra intestinal infections in children [5–7].

In this respect, common antimicrobial classes for which resistance has become a major problem include the β-lactams and fluoroquinolones [8]. Some recent reports from Iran especially for *Escherichia coli* diarrhoeal pathogens have shown that these strains

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exhibited high resistance to ampicillin, erythromycin, cephalothin, co-trimoxazole, tetracycline, and nalidixic acid which are now commonly used in our region [9].

Extended spectrum β-lactamases (ESBLs) as plasmid mediated, TEM and SHV derived enzymes were first isolated from *Klebsiella spp*. and *Escherichia coli* strains. These enzymes are able to effectively hydrolyze broad spectrum cephalosporins and monobactams, but remained partially inactive against some other antibiotics such as cephamycins and imipenem [10]. Widespread use of third generation cephalosporins and aztreonam is believed to be the major cause of mutations in these enzymes which has led to the emergence of the ESBLs [11]. Furthermore, ESBL producing organisms exhibit co-resistance to many other classes of antibiotics, which limits the choice of treatment. Because of the variable affinity of these enzymes for different substrates, identifying organisms which are ESBL producers is a major challenge for the clinical laboratories [12]. The aim of the work was to evaluate antibacterial resistance patterns of clinical isolates of ESBL-producing *EPEC* in a group of Iranian children with and without diarrhoea and to investigate its genetic patterns.

Patients and methods

EPEC isolates

In this prospective study, 192 non-repeats EPEC isolates were collected from stool samples of children with and without diarrhoea. The EPEC strains examined in this study were isolated from 1355 stool specimens obtained from 247 children with diarrhoea (0–10 years old; mean age, 5.5 years) and 1108 children without any gastrointestinal symptoms (0–10 years old; mean age, 6.8 years) during the summer months in three Iranian provinces: Tehran, Ilam and Mazandaran. Strains biochemically identified as *E. coli* were selected and both lactose-positive and lactose-negative *E. coli* strains were identified by the presence of *eaeA* and *bfpA* as EPEC virulence genes. Detection of virulence genes were examined using PCR with the specific primers [13]. The study was approved by the Ethical committee of Hamadan University of Medical Sciences, Hamadan, Iran.

Antimicrobial susceptibility testing of E. coli isolates

Antimicrobial susceptibilities were determined by Kirby Bauer's Method [14,15]. Susceptibility of isolates to antimicrobial agents was determined using commercially available disks (HiMedia Co, India) impregnated with the following antibiotics (drug concentrations in μg): Imipenem (10), gentamicin (10), amikacin (30), ciprofloxacin (5), ampicillin (10), ampicillin-sulbactam (10/10), cefotaxime (30), ceftazidime (30), ceftriaxone (30), cefpodoxime (10), aztreonam (30), tetracycline (30), trimethoprim (10) and chloramphenicol (30). *Escherichia coli* ATCC 25922 were used as the standard quality control strain and results interpenetrate as CLSI guideline M100S [16].

Detection of ESBLs producing strains by double disk method

The 192 EPEC isolates were screened for ESBLs production by the double disk synergy test (DDST) method [17]. DDST was performed by placing disks (MAST Co, England) of ceftazidime, cefotaxime, cefpodoxime, ceftriaxone and aztreonam (30 μg each) at a distance of 20 mm (center to center) from a disk containing ceftazidime/clavulanic acid (30/10 μg), cefotaxime/clavulanic acid (30/10 μg), cefpodoxime/clavulanic acid (30/10 μg), ceftriaxone/-clavulanic acid (30/10 μg) and aztreonam/clavulanic acid (30/10 μg). ESBL production was inferred when the cephalosporin zones were expanded by the clavulanate. A difference of ≥5 mm between the zone diameters of either of the cephalosporin disks and their respective cephalosporin/clavulanate disk is considered to be phenotypic confirmation of ESBL production.

DNA extraction and amplification of genes by PCR

DNA extraction of 192 EPEC isolates was performed as in previous studies, and β-lactamase genes (*blaTEM, blaSHV, blaCTX-M, blaOXA*), and *ISEcp1* were detected by PCR using specific primer pairs listed in Table 1 [17,18]. The oligonucleotides and all reagents for PCRs were synthesized and purchased from Incorporation Bioneer (Daejeon, South Korea). The PCR amplification procedure was performed with 25 μl of master mix containing 0.2 μl of Taq polymerase 5 U/μl, 2.5 μl of 10X PCR buffer along with MgCl2, 1 μl of 10 pM from each reverse and forward primers, 2.5 μl of dNTPs MIX (2 Mm), 3 μl of DNA template, 14.8 μl of DNase and RNase-Free Distilled Water. PCR amplification was done in the thermal cycler device. Agarose gel electrophoresis of the amplified DNA product with 100 bp size marker (Fermentas, South Korea) was carried out in a 2% agarose gel and stained with ethidium bromide.

Statistical analysis

Analytical results were calculated using the Statistical Package for the Social Sciences (SPSS Inc., Chicago, IL, USA) version 16.0 for windows. Differences between values were considered significant at $p \leq .05$.

Results

Assessment of antibiotic resistance and susceptibility patterns of 192 EPEC isolates showed that the highest antibiotic susceptibility was to imipenem (100%), followed by gentamicin (82.3%) and ciprofloxacin (79.2%). The highest resistance was to cefpodoxime (97.9%), trimethoprim (60.7%), and tetracycline (58.4%) (Table 2). Among EPEC isolates, 132 (68.8%) of strains were resistant to three or more classes of antibiotics, which was considered as multidrug resistance (MDR) strains. Regarding susceptibility to different cephalosporins and monobactams determined by DDST method, at least one drug resistance was observed in 97.9% of EPEC strains. The results of the DDST test revealed that among 192 isolated *E. coli* strains, 153 strains (79.7%) were ESBL-producing and 39 (20.3%) were negative for ESBL enzymes. On comparing the antimicrobial resistance patterns, no difference between the negative and positive ESBL-producing EPEC strains was detected (Table 3).

Studying different genes in 192 EPEC strains using PCR showed that 84 (43.8%) isolates were positive for ESBLs encoding genes. The *blaCTX-M* gene was detected in 21 strains (10.93%), *blaSHV* gene in 23 strains (11.97%), *blaTEM* gene in 26 strains (13.54%), *blaOXA* gene in 14 strains (7.29%), and *ISECP1* gene in 61 strains (31.77%). With respect to molecular characteristics of 153 ESBL-

Table 1
Sequences of used primers.

Gene	Primer (5′–3′)	Size bp	References
blaCTX- M	TCTTCCAGAATAAGGAATCCC CCGTTTCCGCTATTACAAAC	909	14
bla SHV	CTTTACTCGCTTTATCG TCCCGCAGATAAATCAC	868	14
bla TEM	ATGAGTATTCAACATTTCCG CCAATGCTTAATCAGTGAGC	931	14
blaOXA	ACACAATACATATCAACTTCG AGTGTGTTTAGAATGGTGATC	813	26
ISEcp1	AAAATGATTGAAAGGTGGT ACTTTACTGGTRCTGCACAT	546	19

Table 2
Antibiotic resistance and susceptibly patterns of EPEC isolates.

Antibiotic	Sensitive No (%)	Intermediate No (%)	Resistance No (%)
Ampicillin (A)	76 (39.6)	27 (14.1)	89 (46.3)
Ampicillin/SULBACTUM (A/S)	95 (49.4)	56 (29.3)	41 (21.3)
Ceftazidime (CA)	143 (74.5)	33 (17.1)	16 (8.4)
Cefotaxime (CE)	90 (46.8)	91 (47.4)	11 (5.8)
Ceftriaxone (CI)	142 (73.9)	50 (26.1)	0 (0.0)
Cefpodoxime (CEP)	0 (0.0)	4 (2.1)	188 (97.9)
Imipenem (I)	192 (100)	0 (0.0)	0 (0.0)
Aztereonam (Ao)	7 (3.7)	154 (80.6)	31 (15.7)
Gentamicin (Gm)	158 (82.3)	23 (11.9)	11 (5.8)
Amikacin (AK)	129 (67.1)	49 (25.6)	14 (7.3)
Tetracycline (TE)	32 (16.7)	48 (24.9)	112 (58.4)
Ciprofloxacin (CF)	152 (79.2)	34 (17.6)	6 (3.2)
Trimethoprime (Tr)	58 (30.1)	18 (9.2)	116 (60.7)
Chloramphenicol (C)	127 (66.1)	29 (15.1)	36 (18.8)

Table 4
Molecular characteristics of 153 ESBL-producing EPEC isolates.

Genotype	ESBL- positive strains No (%)
CTX-M/TEM/SHV/OXA	1 (0.65)
CTX-M/TEM/SHV	4 (2.6)
CTX-M/TEM/OXA	4 (2.6)
CTX-M/SHV/OXA	2 (1.3)
CTX-M/TEM	3 (1.96)
CTX-M/SHV	2 (1.3)
CTX-M/OXA	0 (0.0)
TEM/SHV/OXA	2 (1.3)
TEM/SHV	2 (1.3)
TEM/OXA	0 (0.0)
SHV/OXA	3 (1.96)
CTX-M	11 (7.2)
TEM	14 (9.2)
SHV	12 (7.8)
OXA	7 (4.6)

producing *EPEC*, *SHV* gene of ESBL-producing *E. coli* was detected at 7.8%. Also, *CTX-M* and *TEM* genes were detected in 7.2% and 9.2%, respectively. While, *CTX-M/OXA* and *TEM/OXA* gene subtypes of ESBL-producing *E. coli* were not detectable (Table 4).

Regarding concomitant existence of the ESBL encoding genes and *ISECP1*, *CTX-M* subtypes were detected in 90.5%, *TEM* in 73.0%, *SHV* in 65.0%, and *OXA* in 57.1% of the *ISECP1*, synchronously.

Different antimicrobial resistance patterns were seen in association with the different ESBL encoding genes. The *CTX-M*-positive strains were associated with high rates of resistance to cefpodoxime (100%), aztreonam (66.7%), and ampicillin (66.6%). *SHV* positive strains were associated with the highest resistance rate to cefpodoxime (100%) and trimethoprim (78.2%). Also, *TEM* was associated with the highest resistance rate to cefpodoxime (96.2%) and trimethoprim (65.4%), and *OXA* was associated with the highest resistance rate to cefpodoxime (92.8%) and trimethoprim (71.4%).

Discussion

The problem of antibiotic resistance is present in Iran, where easy and unlimited access to antibiotics reduces the value of such agents. In the current study, 192 EPEC strains isolated from children revealed all isolates to be sensitive to imipenem, which is similar to a study previously conducted in Iran [3,13] as well as from other countries [19–21], while 31.3% were resistant to more than one classes of antibiotics and 68.8% were MDR, which is thus higher to previous reports from our region [13,22]. In our research, high levels of resistance to commonly used antibiotics such as ampicillin and co-trimoxazole were observed in EPEC isolates. This result is in accordance with previous findings from other studies [22–24]. The resistance of EPEC isolates to trimethoprim (60.7%) was more than other research (17.8%) reported from Brazil [25]. Compared with other investigations [13,22], our study showed a lower prevalence of resistance to ciprofloxacin in EPEC isolates. However, in other reports, almost all of the isolates were found to be resistant to ciprofloxacin, trimethoprim and tetracycline [26].

We found the susceptibility to different types of cephalosporin lower than that reported in other countries [20,21,26]. Also, the present study showed that the resistance rates to chloramphenicol, tetracycline, gentamicin, trimethoprim, and amikacin ranged between 16.7% and 67%, while this range was wider in other studies (7% to 69%) [20,21,27,28]. Generally, it seems that in the cephalosporin group, EPEC isolates are most resistant to cefpodoxime, and most sensitive to ciprofloxacin. Among other antibiotic groups, the highest susceptibility was observed to gentamicin. The widespread use of antibiotics plays an important role in the emergence of resistant bacteria. Although there were low levels of preexisting antibiotic-resistant bacteria before the widespread use of antibiotics [29,30], evolutionary pressure from their use has played a role in the development of multi-drug resistance organisms and the spread of resistance between bacterial species [31,32]. The major problem of the emergence of resistant bacteria is due to misuse and overuse of antibiotics by health practitioners as well as patients.

DDST test showed that 153 strains (79.7%) were ESBL-producing. This result is in accordance with previous findings

Table 3
Antibiotic resistance pattern of ESBL (+) and ESBL (−) isolates by DDST method.

Antibiotic	ESBL (−) (n = 39)			ESBL (+) (n = 153)			P-value
	S N (%)	I N (%)	R N (%)	S N (%)	I N (%)	R N (%)	
Ampicillin (A)	15 (38.4)	6 (15.4)	18 (46.2)	61 (39.9)	22 (14.4)	70 (45.8)	.864
Ampicillin/Sulbactam (A/S)	20 (51.3)	11 (28.2)	8 (20.5)	75 (49.1)	45 (29.4)	33 (21.6)	.806
Ceftazidime (CA)	29 (74.4)	7 (17.9)	3 (7.7)	114 (74.5)	26 (17.0)	13 (8.5)	.990
Cefotaxime (CE)	18 (46.2)	19 (48.7)	2 (5.1)	72 (47.0)	72 (47.0)	9 (6.0)	.929
Ceftriaxone (CI)	29 (74.4)	10 (25.6)	0 (0)	113 (73.9)	40 (26.1)	0 (0)	.949
Cefpodoxime (CEP)	0 (0)	1 (2.6)	38 (97.4)	0 (0)	3 (1.9)	150 (98.1)	.783
Imipenem (I)	39 (1 0 0)	0 (0)	0 (0)	153 (1 0 0)	0 (0)	0 (0)	.999
Aztereonam (Ao)	1 (2.6)	32 (82.0)	6 (15.4)	6 (3.9)	123 (80.4)	24 (15.7)	.699
Gentamicin (Gm)	32 (82.0)	5 (12.8)	2 (5.2)	126 (82.4)	18 (11.8)	9 (5.8)	.953
Amikacin (AK)	27 (69.2)	10 (25.6)	2 (5.2)	102 (66.7)	39 (25.5)	12 (7.8)	.767
Tetracycline (TE)	16 (41.0)	10 (25.6)	13 (33.4)	26 (17.0)	38 (24.8)	89 (58.2)	.360
Ciprofloxacin (CF)	31 (79.5)	7 (17.9)	1 (2.6)	121 (79.1)	27 (17.6)	5 (3.3)	.956
Chloramphenicol (C)	26 (66.7)	5 (12.8)	8 (20.5)	102 (66.7)	23 (15.0)	28 (18.3)	.753

[24] but are in contrast with other reports [22]. ESBL-producing strains were comparable with the non ESBL-producing isolates with regard to the susceptibility to the antibiotics. Although previous studies have shown the association of ESBL genes with antibiotic resistance, no significant association was observed between these two categories in this study.

In various populations, resistance rate as well as frequency of detected ESBL-encoding genes were different in *E. coli* isolates. In the present study, 79.7% of *EPEC* isolates were classified as ESBL producers based on phenotypic detection of ESBLs. Analysis of the ESBLs-encoding genes indicated that the 84 (54.9%) strains harboured at least one ESBLs gene and majority of the ESBL-positive isolates harbored SHV (15.0%) followed by CTX-M (13.7%), TEM (17.0%), and finally, OXA (9.2%) as sole gene. Data on ESBLs in EPEC isolates are not available. In laboratories, there are little attempts to diagnose and determine antibiotic resistance patterns of these bacteria. Thus, documents on the resistance of EPEC are not routinely produced, as for other bacterial pathogens.

In conclusion, considering that 79.7% of EPEC strains isolated from Iranian children were ESBL-producing, the performance of antibiogram test is necessary before antibiotic therapy for complete treatment and prevention of diarrhoea caused by multi-drug resistant agents.

Conflict of interest

The authors declare that there are no conflicts of interest.

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