Gastroenterology and Hepatology From Bed to Bench.

ORIGINAL ARTICLE

Extended spectrum betalactamase producing Enteroaggregative *Escherichia coli* from young children in Iran

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ABSTRACT

Aim: The aim of this study was to investigate the frequency of betalactamase producing EAEC isolates among young children with diarrhea in Zanjan, Iran.

Background: Entero aggregative *Escherichia coli* (EAEC) is an emerging enteric pathogen associated with acute and persistent diarrhea and the evolution and spread of acquired extended spectrum betalactamases (ESBLs) among these strains has become a serious problem in the management of infectious diseases in developing countries.

Patients and methods: During the period from March 2011 to January 2012, 140 isolates of *E. coli* from diarrheal children aged 0–60 months and 90 isolates from age-matched controls without diarrhea were investigated for EAEC using PCR. Antimicrobial susceptibility testing was performed as CLSI guidelines and betalactamase genes, including bla_{TEM}, bla_{CTX-M}, bla_{IMP}, bla_{VIM} and bla_{NDM-1} investigated in EAEC isolates.

Results: In this study, EAEC was detected with slightly higher frequency in children with (8%) than in children without (4.6%) diarrhea (P>0.05). Diarrheagenic *E. coli* exhibited high level resistance to aztreonam (80.7%), amoxicillin (74.4%) and tetracycline (69.3%). Also, 86.4% of *E. coli* isolates were resistant to at least three different classes of antimicrobial agents and considered as multidrug resistance. Molecular characterization of betalactamase genes showed that bla_{TEM} was the most frequently isolated betalactamase. It was detected in 78.9% of ESBL producing EAEC isolates. Also, the frequency of bla_{CTX-M} was 63.1% (12/19) and 8 (42.1%) isolates carried the bla_{TEM} and bla_{CTX-M}, simultaneously. None MBL producing EAEC was detected in our study.

Conclusion: Our results indicate that ESBLs especially bla_{TEM} and bla_{CTX-M} are widespread among EAEC isolates and appropriate surveillance and control measures are essential to prevent further dissemination of betalactamases in our country.

Keywords: Antibiotic resistance, Diarrhea, EAEC, ESBL.

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Introduction

Enteroaggregative *Escherichia coli* (EAEC) is a subgroup of diarrheagenic *E. coli* (DEC) and has emerged as an important pathogen in travellers' diarrhea, diarrhea among children and in immunocompromised patients (1, 2). EAEC are associated with watery diarrhea among children younger than five years in developing countries and represent a major public health problem in these areas (3, 4). Nguyen and colleagues, in a study of children younger than 5 years in Vietnam, identified EAEC in 11.6% of diarrheal children, compared with 4.4% of age-matched controls

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without diarrhea (1). A PCR method based on the presence of essential virulence factors as anti-aggregation protein transporter (CVD432 or the AA probe) would improve the diagnosis of EAEC diseases (5, 6).

The continuous emergence of resistance to antimicrobial agents among the prevalent pathogens is the most dangerous threat for the treatment of infectious disease (6). Compared with other diarrheagenic *E. coli*, EAEC was found to be highly resistant to many commonly used antimicrobials agents (7). The majority of the EAEC from diarrheal patients in Kolkata, India, exhibited multidrug resistance including resistant to fluoroquinolones (8). Furthermore, *E. coli* isolates resistant to oxyiminocephalosporins due to the production of extended spectrum betalactamases (ESBL) have emerged worldwide (9). During the next few years, CTX-M has become the predominant ESBL family and CTX-M-producing *E. coli* has spread globally and has been involved in nosocomial outbreaks and community acquired infections (10, 11). The acquisition of resistance genes by horizontal transfer is currently thought to play a major role in the development of multidrug resistant (MDR) strains (12, 13). Regular surveillance of antibiotic resistance provides information for antibiotic therapy and resistance control (6).

The objectives of the present study were (1) to determine the frequency of EAEC among children younger than 5 years with and without diarrhea and (2) to investigate pattern of antimicrobial resistance and frequency of betalactamase genes including $bla_{\text{CTX-M}}$, bla_{TEM}, bla_{IMP}, bla_{VIM} and $bla_{\text{NDM-1}}$ among EAEC isolates in Zanjan, Iran.

Patients and Methods

This study included 600 stool specimens from children younger than five years of age between March 2012 and February 2013. Patients included 450 children with and 150 without diarrhea attending four major university hospitals in Zanjan, Iran. Control subjects were healthy children with no history of diarrhea and antibiotic therapy for at least 1 month. Stool samples collected in Cary-Blair transport medium were cultured on MacConkey agar (Merck, Germany) and then identified by standard biochemical methods. Verified isolates were preserved at –70 °C in Tripticase soy broth (Merck, Germany) containing 20% (v/v) glycerol for further analysis.

Antimicrobial susceptibility testing and phenotypic characterization

Susceptibility of isolates to the following antibiotics was examined using the disk diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (14): Amoxicilin (25µg), Aztreonam (30µg), Amikacin (30µg), Cefotaxime (30µg), Cefoxitine (30µg), Ceftazidime (30µg), Ciprofloxacin (5µg), Co-amoxiclav (30µg), Co-trimoxazole (25µg), Erythromycin (25µg), Gentamicin (10µg), Imipenem (10µg) and Tetracycline (30µg) (MAST, UK). Isolates shown to be resistant to at least three different classes of antimicrobial agents were determined to be multidrug resistant (MDR). *Escherichia coli* ATCC 25922 was used as control for antibiotic resistance. Phenotypic characterization of extended spectrum betalactamases (ESBLs) and metallo betalactamase (MBLs) was determined using Double Disk Synergy Test (DDST) according to CLSI criteria.

Detection of Enteroaggregative *E. coli* and betalactamases by PCR

Enteroaggregative *E. coli* was detected by virulence markers *pCVD432* (the nucleotide sequence of the EcoRI-PstI DNA fragment of pCVD432) and *astA* (enteroaggregative heat stable toxin). Furthermore, ESBL or MBL producing isolates were tested for *bla* genes including bla_{TEM}, $bla_{\text{CTX-M}}$, bla_{IMP}, bla_{VIM} and $bla_{\text{NDM-1}}$ using the primers listed in Table 1.

Table 1. Primers used in this study

Target	Primer sequence (5'→3')	Amplicon size (bp)	Ref.
bla_{TEM}	TCCGCTCATGAG ACA ATA ACC TTCGTCTGACAGTTACCAATGC	931	Kiratisin et al. 2008
bla_{CTX-M}	GGTTAAAAAATCACTGCGTC TTGGTGACGATTTTAGCCGC	909	Kiratisin et al. 2008
bla_{IMP}	GGAATAGAGTGGCTTAATTCTC CCAAACCACTACGTTATCT	188	Ellington et al. 2007
bla_{VIM}	GATGGTGTTTGGTCGCATA CGAATGCGCAGCACCAG	390	Ellington et al. 2007
pCVD432	CTGGCGAAAGACTGTATCAT AAATGTATAGAAATCCGCTGTT	630	Aslani et al. 2011
astA	CCATCAACACAGTATATCCGA GGTCGCGAGTGACGGCTTTGT	111	Aslani et al. 2011
NDM-1	ACCGCCTGGACCGATGACCA GCCAAAGTTGGGCGCGGTTG	263	Shahcheraghi et al. 2013

The total DNA was extracted from whole organisms by boiling. The PCR mixtures with a final volume of 25 µl consisted of 5 µl template DNA; 0.2 mM of each deoxynucleoside triphosphate; 10 pmol of each primers; 10 mM Tris- HCl; 1.5 mM MgCl2; 50 mM KCl; 1.5 U of Taq DNA polymerase. PCR was performed with the Gene Atlas 322 system (ASTEC, Japan). Amplification involved an initial denaturation at 94°C, 5 min followed by 35 cycles of denaturation (94°C, 50 s), annealing (50°C, 1 min for blaTEM, blaCTX-M, blaVIM and blaIMP, 52°C, 40 s for astA, 55°C, 40 s for pCVD432 and 58°C, 50 s for blaNDM-1) and extension (72°C, 1 min), with a final extension step (72°C, 8 min). The amplified DNA was separated by submarine gel electrophoresis on 1% agarose, stained with ethidium bromide, and visualized under UV transillumination. The following reference strains were used as positive and negative controls: EAEC 97R (pCVD432), E. coli ATCC 35218 (blaTEM), K. pneumoniae 7881 (blaCTX-M), A. baumannii AC54/97 (blaIMP), E. coli K12 DH5α (no virulence gene) and E. coli ATCC 25922 (non betalactamase produser).

Statistical analysis

The data were analyzed with SSPS version 17.0 software (SPSS, Inc., Chicago, IL). The chi-square test was used to determine the statistical significance of the data. A P value of < 0.05 was considered significant.

Results

Detection and characterization of Enteroaggregative *E. coli* from clinical stool samples

A total of 450 children with diarrhea and 150 control children without diarrhea were studied. Among the total children, 133 (22.1%) children were younger than 12 months, 180 (30 %) were 13–24 months and 287 (47.9 %) were 25–60 months. The mean of age in patient and control groups was 24 and 20 month, respectively. The sex distribution was 364 (60.7%) male and 236 (39.3 %) female.

Overall, 230 (38.3%) *E. coli* isolates were identified in the 600 stool samples: 140 isolates from diarrheal patients and 90 isolates from control group. The frequency of EAEC in the diarrheal patients and healthy controls was 36 (8%) and 7 (4.6%) isolates, respectively. EAEC isolates were identified with slightly higher frequencies in diarrheal patients than in control group ($P > 0.05$). Out of the 36 EAEC isolates harboring the *pCVD432*, 12 (2.7%) also had the *astA* gene.

Table 2. Antimicrobial susceptibility of EAEC and Non EAEC isolates

Antimicrobial agents	EAEC (n= 36) (%)			Non-EAEC (n= 104)(%)			Total DGEC (n= 140) (%)		
	R	I	S	R	I	S	R	I	S
Erythromycin	36(25.7)	0	0	104(74.3)	0	0	140 (100)	0	0
Amoxicillin	26(18.6)	1(0.7)	9(6.4)	78(55.7)	3(2.7)	23(16.4)	104(74.4)	4(2.8)	32(22.8)
Aztreonam	3(04)	2(1.4)	4(2.8)	83(59.3)	17(12.1)	4(2.8)	113(80.7)	19(13.6)	8(5.7)
Co-amoxiclav	31(22.1)	1(0.7)	4(2.8)	69 (49.3)	5(3.6)	30(21.4)	100(71.4)	6(4.3)	34 (24.3)
Cefotaxime	14(10)	10(7.1)	12(8.6)	57(40.7)	9(6.4)	38(27.1)	71(50.7)	19(13.6)	50 (35.7)
Ceftazidime	21(15)	9(6.4)	6(4.3)	67(47.8)	18(12.8)	19(13.6)	88(62.8)	27(19.4)	25 (17.8)
Cefoxitine	13(9.3)	1(0.7)	22(15.7)	32(22.8)	6(4.3)	66(47.1)	45(32.1)	7(5)	88 (62.9)
Co-trimoxazole	8 (5.7)	14(10)	14 (10)	22(15.7)	8(5.7)	74(52.8)	30(21.5)	22(15.7)	88(62.9)
Ciprofloxacin	17(12.1)	5(3.6)	14(10)	35(25)	22(15.7)	47(33.6)	52(37.1)	27(19.3)	61 (43.6)
Tetracycline	24(17.1)	9(6.4)	3 (9.3)	73(52.1)	24(17.1)	7 (5)	97(69.3)	33(23.6)	10 (7.1)
Amikacin	2(1.4)	15(10.7)	19(13.6)	28(20)	20(14.3)	56 (40)	30(21.4)	35(25)	75 (53.6)
Gentamicin	15(10.7)	12(8.6)	9 (6.4)	26(18.6)	38(27.1)	40(28.6)	41(29.3)	50(35.7)	49 (35)
Imipenem	1(0.7)	5(3.6)	30(21.4)	2(1.4)	30(21.4)	72(51.4)	3(2.1)	35(25)	102 (72.9)

Antimicrobial susceptibility testing

Antimicrobial susceptibility of EAEC and non-EAEC strains isolated from diarrheal patients is presented in Table 2 and Fig 1. The highest rate of resistance among diarrheagenic *E. coli* showed against to Erythromycin (100%) followed by aztreonam (80.7%) and amoxicillin (74.4%). Although, imipenem resistance rate was 2.1% (3 isolates), but the intermediate resistant isolates (25%) should be concerned. A total of 121 (86.4%) isolates of *E. coli* were multidrug resistant (MDR). Out of the 36 EAEC isolates, 19 (52.7%) were ESBL positive, whilst none imipenem resistant and MBL producing EAEC was detected.

Molecular characterization of ESBL and MBL genes

EAEC isolates were subjected to PCR experiments to detect betalactamase genes, including bla_{TEM}, bla_{CTX-M}, bla_{IMP}, bla_{VIM} and bla_{NDM-1}. Bla_{TEM} was the most frequently isolated betalactamase. It was detected in 78.9% (15/19) of ESBL producing EAEC isolates. The frequency of bla_{CTX-M} was 63.1% (12/19) and 8 (42.1%) isolates of EAEC producing ESBL carried the bla_{TEM} and $bla_{CTX-M,}$ simultaneously. None MBL producing EAEC was detected in our study.

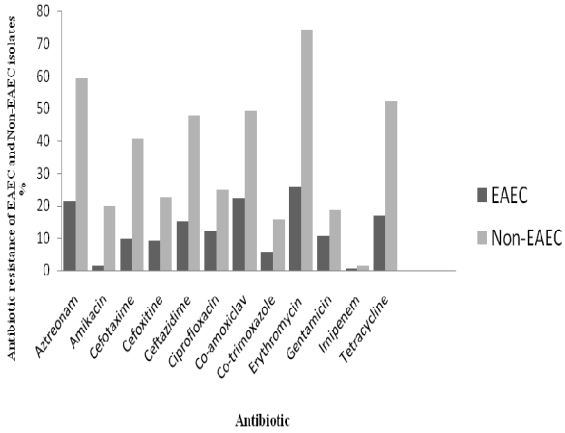

Figure 1. Antibiotic resistance of EAEC and non EAEC isolates

Discussion

EAEC is an emerging enteric pathogen associated with acute and persistent diarrhea (≥ 14 days) and may cause malnutrition and growth defects in children. It has been identified in traveller's diarrhea in both developing and developed countries and has been isolated in immunocompromised patients (15, 16). An increasing number of studies support the association of EAEC with diarrhea in populations in developing

countries, most prominently in association with persistent diarrhea. In several studies, culture of EAEC from the stool during the first few days of diarrhea is predictive of a longer duration of illness. The association of EAEC with diarrhea appears to vary geographically, and many studies have demonstrated the importance of EAEC in pediatric diarrhea. In studies carried out in Vietnam and the USA, EAEC was isolated at higher prevalence in children with diarrhea (11.6 and 4.5%, respectively) than in controls (7.2 and 1.7%, respectively) (1, 17, 18). Our findings, however, were in contrast to these studies. According to results, EAEC pathotype isolated in both children with and without diarrhea and there was no significant association between EAEC and diarrhea. The prevalence of this pathotype in diarrheal and healthy children's was 8% and 4.6%, respectively. This lack of association with diarrhea has been observed in another study. In a study carried out in Nicaragua, EAEC strains were the most frequently isolated pathotype of *E. coli*; however, the isolation rate as a total was slightly higher in the healthy group than in the diarrheal group. It has also been shown that EAEC is a heterogeneous group of *E. coli* and not all strains are capable of causing diarrhea (19). Among the few published studies in Iran, only Salmanzadeh-Ahrabi et al has reported the association of EAEC isolates with diarrhea. They reported EAEC isolates in 24% of childrens with diarrhea and 8% of controls ($p<0.0001$) (20, 21).

Antimicrobial resistance and the spread of betalactamases among human pathogens has become a major public health problem in developing countries. In a study carried out in Kolkata, India, majority of EAEC isolates from diarrheal patients exhibited multidrug resistance including resistant to fluoroquinolones (8). Peruvian EAEC isolates were also multidrug resistant, especially to ampicillin, cotrimoxazole, tetracycline and nalidixic acid (22). According to results, EAEC isolates exhibited high level resistance to various antibiotics. The high frequency of antibiotic resistant isolates of EAEC may be due to the widespread use of numerous antimicrobial agents in our country. Furthermore, a majority of isolates were resistant to aztreonam, amoxicillin and tetracycline, resulting in a high percentage of multidrug resistance.

In Asia, the frequency of ESBL-positive *Enterobacteriaceae* has been shown to vary in different countries. National survey data have indicated the prevalence of ESBLs in 5 to 8% of *E. coli* isolates from Korea, Japan, Malaysia and Singapore but in 12-24% in Thailand, Taiwan, the Philippines, Indonesia, Hong Kong and China (23). In the present study, the frequency of ESBL positive isolates of EAEC was 52.7%. The frequency of bla_{TEM} and $bla_{CTX\text{-}M}$ in EAEC isolates was 78.9% and 63.1%, respectively. Metallo betalactamases have been reported from many countries, particularly in multidrug resistance pathogens like *Pseudomonas aeruginosa* and Acinetobacter species. None MBL producing EAEC was detected in our study. Our results indicate that ESBLs especially bla_{TEM} and $bla_{CTX\text{-}M}$ are widespread among EAEC isolates and appropriate surveillance and control measures are essential to prevent further dissemination of betalactamases in our country.

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