

## Identification and characterization of class 1 integrons among atypical enteropathogenic *Escherichia coli* isolated from children under 5 years of age

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### ABSTRACT

**Background and Objectives:** The therapeutic options for diseases caused by *Escherichia coli* are limited. In this study we investigated the presence of virulence factors among Enteropathogenic *Escherichia coli* (EPEC) strains and their antibiotic resistance patterns. The isolates were also checked for the presence of class 1 integrons and gene cassettes.

**Materials and Methods:** This study included 70 EPEC strains isolated from children. Antimicrobial resistance patterns were determined using diffusion methods. The broth microdilution methods was used to determine the minimum inhibitory concentration. PCR was used to detect *eaeA*, *bfpA* genes. The 5' and 3' conserved sequences (CSs) of class 1 integrons and *intI* gene were amplified to investigate the presence of integrons and gene cassettes.

**Results:** Antimicrobial susceptibility testing showed that 4 (5.7%), 3 (4.2%), and 2 (2.8 %) isolates were resistant to ampicillin, trimethoprim-sulfamethoxazole, and ceftazidime, respectively. Resistance rates to ciprofloxacin and aztreonam were 1.4%. Thirteen (18.5%) isolates showed resistance to tetracycline, and 4 (5.7%) were kanamycin resistant. Class I integron detected in 22 (31.4%) isolates. All the gene cassettes found in class I integrons corresponded to different variants of *dfr* and *aadA* genes.

**Conclusion:** Prevalence of class I integrons in EPEC strains was high. Presence of *aadA* and *dfr* gene cassettes in integrons represents high distribution of resistance determinants in EPEC strains.

**Keywords:** Class I integrons, Enteropathogenic *Escherichia coli*, Children

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### INTRODUCTION

Diarrhea remains one of the major causes of morbidity and mortality among children worldwide (1). Enteropathogenic *Escherichia coli* (EPEC) is the predominant cause of diarrhea in infant worldwide and represents an important endemic health threat to children under 5 years of age in developing countries

(2). Approximately 30-40% of infant diarrhea is caused by EPEC (3). In Iran, EPEC strains were isolated from 44.9% of the children with diarrhea (4). These strains possess a number of virulence factors and a virulence plasmid that are responsible for infections caused by EPEC, although they cannot produce classic toxins (5).

EPEC is classified into two groups: Typical EPEC (tEPEC), and atypical EPEC (aEPEC). The main virulence mechanism of EPEC is localized adherence to the host gut epithelium by inducing of a specific lesion on enterocytes, which called attaching-effacing (A/E) lesion. The locus of enterocyte effacement (LEE) encoded adhesion determine establishment of A/E lesions. LEE is a chromosomal pathogenicity

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**Table 1.** Oligonucleotide primers used in this study.

Primer	Target gene/ region		Sequence (5'-3')	Product size (bp)	Annealing temp (°C)	Reference
BFP-F	<i>bfpA</i>		AATGGTGCTTGCCTTGCTGC	326	56	36
BFP-R			GCCGCTTTATCCAACCTGGTA			
EAE-F	<i>eaeA</i>		CATTATGGAACGGCAGAGGT	790	55	37
EAE-R			ATCTTCTGCGTACTGCGTTCA			
CS-F	5	CS	GGCATCCAAGCAGCAAG	Variable	54	38
CS-R			3			
Int-F	<i>intI</i>		GCCTTGCTGTTCTTCTACGG	558	56	39
Int-R			GATGCCTGCTGTTCTACGG			
SUL-F	<i>sul-1</i>		CTTCGATGAGAGCCGGCGGC	437	68	38
SUL-R			GCAAGGCGGAAACCCGCGCC			
qac-F	<i>qacEA1</i>		ATCGCAATAGTTGGCGAAG	240	54	39
qac-R			CAAGCTTTTGCCCATGAAGC			

island, which is present in both EPEC subgroups (6).

Recently, therapeutic options for diseases caused by *E. coli* are limited because of the emergence and spread of Extended Spectrum  $\beta$ -lactamases (ESBL), and increasing of multiple drug resistant strains (7). The carbapenems are sometimes the only effective options against resistant strains. However, recent studies reported carbapenems resistance in clinical isolates of *E. coli*, which is a major cause for concern in treatment of both intestinal and urinary tract infections (8, 9). Integrons play a major role in the antimicrobial resistance of clinical isolates because they are genetic elements capable to capture, integrate and express antimicrobial resistance gene cassettes (10). The efficacy of integrons in distribution of antimicrobial resistance in clinical isolates of *E. coli* has been well documented (11, 12). In this study, we investigated the prevalence of EPEC among Iranian children and study their resistance patterns and presence of class1 integrons in EPEC strains.

## MATERIAL & METHODS

### Bacterial strains and identification methods.

This study included 70 EPEC strains isolated from children less than 5 years of age with and without diarrhea in Tehran hospitals from May 2010 to July 2012. All isolates were identified using standard microbiological methods (4).

**Identification of *eaeA* and *bfpA* gene.** DNA of bacterial isolates was extracted as described previously

(13). All isolates were tested by PCR to detect *eaeA* and *bfpA* gene in EPEC strains. Primers and PCR conditions used in this study are listed in Table 1.

**Serogrouping of EPEC Strains.** Determination of EPEC serogroups was performed by slide agglutination method with O-specific antisera according to the instructions recommended by manufacturer (Bio-Rad, France).

**Antimicrobial Susceptibility testing.** Antimicrobial susceptibility pattern of strains was determined by disk diffusion method, according to the Clinical Laboratory Standard Institute (CLSI) criteria (14). The following antimicrobial discs were examined: ampicillin (30  $\mu$ g/ml), aztreonam (30  $\mu$ g/ml), cefotaxime (30  $\mu$ g/ml), cefepime (30  $\mu$ g/ml), ceftazidime (30  $\mu$ g/ml), ciprofloxacin (5  $\mu$ g/ml), gentamicin (10  $\mu$ g/ml), amikacin (30  $\mu$ g/ml), kanamycin (30  $\mu$ g/ml), tetracycline (30  $\mu$ g/ml), chloramphenicol (30  $\mu$ g/ml), nalidixic acid (30  $\mu$ g/ml), and trimethoprim-sulfamethoxazole (15  $\mu$ g/ml) (MAST diagnostics, Bostle, Mersey side). In addition, Minimum Inhibitory Concentration (MIC) of kanamycin and tetracycline were determined using broth microdilution as methods recommended by CLSI guidelines (15). *E. coli* ATCC 25922 was used as a susceptible control strain in all antimicrobial testing. Multidrug resistance was defined as resistance to three or more of different antimicrobial classes.

### Detection and characterization of Integrons

**Table 2.** Antimicrobial susceptibility patterns of EPEC strains.

Antibiotics	Resistance	Intermediate	Susceptible
Gentamicin	0(0%)	2(2.18%)	68(97.1%)
Kanamycin	4(5.7%)	19(27.1%)	47(67.1%)
Tetracycline	13(18.5%)	16(22.8%)	41(58.5%)
Ciprofloxacin	1(1.4%)	0(0%)	69(98.5%)
Nalidixic Acid	1(1.4%)	3(4.2%)	66(94.2%)
Chloramphenicol	2(2.8%)	3(4.2%)	65(92.8%)
Trimethoprim-sulfamethoxazole	3(4.2%)	1(1.4%)	66(94.2%)
Ceftazidime	2(2.8%)	2(2.8%)	66(94.2%)
Cefotaxime	0(0%)	1(1.4%)	69(98.5%)
Aztreonam	1(1.4%)	0(0%)	69(98.5%)
Amikacin	2(2.8%)	0(0%)	68(97.1%)
Ampicillin	4(5.7%)	0(0%)	66(94.2%)
Cefepime	0(0%)	0(0%)	70(100%)

**Table 3.** Characterization of Class I integrons and gene cassettes in 22 Enteropathogenic *Escherichia coli* (EPEC).

STRAINS	Serogroup	H Type	EPEC Type	intI1	qac+ sul1	Size of gene cassettes (bp)	Identified gene Cassettes
16	O111	H4	atypic	+	+	1800	<i>aadA1, dfrA1</i>
17 <sup>b</sup>	O111	NT <sup>a</sup>	atypic	+	+	2000	<i>aadA2, dfrA12, orfF</i>
18 <sup>b</sup>	O111	H21	atypic	+	+	2000	<i>aadA2, dfrA12, orfF</i>
19 <sup>b</sup>	O111	NT	atypic	+	+	1800	<i>aadA1, dfrA1</i>
20	O111	H36	atypic	+	+	1800	<i>aadA1, dfrA1</i>
27	O111	H15	atypic	+	+	1800	<i>aadA1, dfrA1</i>
28	O111	H27	typic	+	+	1800	<i>aadA1, dfrA1</i>
29	O111	H31	atypic	+	+	1800	<i>aadA1, dfrA1</i>
30	O111	H27	atypic	+	+	1800	<i>aadA1, dfrA1</i>
34	O111	H21	atypic	+	+	1800	<i>aadA1, dfrA1</i>
35	O111	H2	atypic	+	+	1800	<i>aadA1, dfrA1</i>
39	O111	H3	atypic	+	+	800	<i>dfrVII</i>
40	O111	H20	atypic	+	+	800	<i>dfrVII</i>
48	O111	H14	atypic	+	+	800	<i>dfrVII</i>
78	O111	NT	atypic	+	+	1800	<i>aadA1, dfrA1</i>
80	O127	NT	atypic	+	+	1800	<i>aadA1, dfrA1</i>
82	O127	NT	atypic	+	+	1800	<i>aadA1, dfrA1</i>
91	O55	H48	atypic	+	+	800	<i>dfrVII</i>
187	O142	H21	atypic	+	+	1500	<i>drfA1, orfC</i>
190	O142	H2	atypic	+	+	800	<i>dfrVII</i>
386	NT	NT	atypic	+	+	1500	<i>drfA1, orfC</i>
1719 <sup>b</sup>	NT	H48	atypic	+	+	1500	<i>drfA1, orfC</i>

<sup>a</sup> NT, Non Typable<sup>b</sup> Multidrug resistance strains (resistance to three or more of different antimicrobial classes).



**Fig 1.** Detection of *int1* gene among nine EPEC strains. Lane M, 100 bp ladder; Lane 1, Positive control strain; Lanes 2 to 10, EPEC strains isolated in this study; Lane N, negative control (*E.coli* ATCC 25922 ).

**and associated gene cassette.** Class I integrons and associated sulfonamide resistance gene (*sul1*), and *int1* region of the *integrase* gene were identified by PCR using primers and PCR conditions listed in Table1. Gene cassette assortments in class I integrons were detected by PCR using 5' and 3' CS primers. The PCR products were sequenced in both directions. Sequences were analyzed via the BLAST and MEGA programmers.

**Nucleotide sequence accession numbers.** The nucleotide sequences of gene cassettes reported in this study have been submitted to GenBank under accession numbers JX442969-72.

## RESULTS

All isolates were confirmed as *E. coli* using routine biochemical identification methods. Presence of *eaeA* gene was confirmed in all 70 strains isolated from diarrhea children. Two isolates (2.85%) had both virulence factors (*eaeA*, *bfpA*) and classified as typical EPEC. However, *bfpA* gene was not found in 68 (97.14%) isolates. These isolates (*eaeA*<sup>+</sup>, *bfpA*<sup>-</sup>) classified as atypical EPEC. H typing of EPEC strains showed 15 different H types. H21 was the most prevalent (8.5%) among EPEC strains isolated from children, followed by H2 (5.7%) and H48 (4.2%).

Resistance to tetracycline (18.5%), ampicillin (5.7%), kanamycin (5.7%), and trimethoprim-sulfamethoxazole (4.2%) was remarkable. However, all isolates were susceptible to gentamicin and cefepime (Table2). Determination of MICs to tetracycline and kanamycin showed that 13 (18.5%) isolates were tetracycline resistant (MIC<sub>50</sub> ≥16 µg/ml) and 4 (5.7%) isolates were kanamycin resistant (MIC<sub>50</sub> ≥64 µg/ml).

Four strains (5.7%) were multidrug resistant (resistance to three or more antimicrobial classes). Class I integron containing *int1*, *qacΔE*, and *sul1* were detected in 22 (31.4%) of strains (Fig.1, Table 3).

Following PCR amplification of variable region of class I integrons, 4 different groups of gene cassette were detected. Groups 1, 2, 3, and 4 included fragments with 2000, 1800, 1500, and 800 bp size, respectively. Characterization of these gene cassettes by sequencing demonstrated different variants of *aadA* (*aadA1*, *aadA2*) and *dfr* genes (*dfrA1*, *dfr7*, *dfrA12*).

Group 1 consisted from two isolates. Three gene cassettes (*dfrA12*, *orfF*, and *aadA2*) were shown in these isolates following sequencing. Twelve isolates with *dfrA1* and *aadA1*, classified in group 2. Group 3 included *dfrA1* and *orfC* gene cassettes that three isolates belonged to this group. Finally five isolates classified in group 4 and were contained *dfr7*.

## DISCUSSION

Enteropathogenic *Escherichia coli* (EPEC) is a subgroup of diarrheagenic *E. coli* that is responsible for infantile diarrhea worldwide, mostly in developing countries (5). In this study, among 70 EPEC strains, just 2 strains (2.8%) belonged to tEPEC. However, 68 strains (97.1%) classified as aEPEC. These result show high frequency of aEPEC strains among children in Iran. This finding agrees with previous studies which have been demonstrated prevalence of aEPEC in children with or without diarrhea worldwide (16-19). Some aEPEC strains could be tEPEC or EHEC strains that lost BFP-encoding plasmid or *stx* genes (20). Furthermore, some adhesion encoding genes (*lpfA*, *paa*, *iha*, and *toxB*) from EHEC have detected in EPEC strains and these genes have been more prevalent in aEPEC than tEPEC (20). Some studies have shown that there are not any relationships between aEPEC and other pathogen types of *E. coli* (21). On the other hand, a closer relationship was found between aEPEC and EHEC (22).

To our knowledge, studies of frequency of antimicrobial resistance in EPEC strains are very rare. High level of antimicrobial resistance have reported in *E. coli* and other *Enterobacteriaceae* strains worldwide (7, 9, 23), but in this study the highest rate of antibiotic resistance were shown in 4 strains (5.7%) that were multiple drug resistance.

Cefepime showed excellent antimicrobial activity against EPEC strains with 100% of all isolates

susceptible. Of other Cephalosporins, ceftazidime and cefotaxime demonstrated good activity (susceptibility rates of 94.2% and 100%, respectively). These trends are in agreement with previous studies that majority of EPEC isolates have been susceptible to third generation cephalosporins (17, 24, 25).

World Health organization has recommended ampicillin and co-trimoxazole as therapeutic options for treatment of severe cases of diarrhea. In this study, 4 (5.7%), and 4 (5.7%) isolates were identified which were unsusceptible to ampicillin and co-trimoxazole, respectively. In a study in Mexico, 38% of aEPEC isolates were resistant to ampicillin and co-trimoxazole but 100%, and 67% of tEPEC were resistant to ampicillin and co-trimoxazole, respectively (17). In contrast, Aslani *et al.* (26) have reported resistant rates of 59%, and 35% for ampicillin and co-trimoxazole, respectively. Similar to earlier published data (24-26), in current study, the majority of isolates (98.5%) were susceptible to ciprofloxacin. However, one isolates was resistant to ciprofloxacin. Furthermore, this isolate was a multiple drug resistant that was resistant to aztreonam, kanamycin, tetracycline, ampicillin, co-trimoxazole, and ceftazidime. Therefore, it seems that aEPEC are more susceptible to ampicillin, co-trimoxazole and other antimicrobial agents than tEPEC.

As EPEC is an important cause of diarrhea in children in developing countries, spread of integrons among these strains could threaten the usefulness of antibiotic therapy in persistent or sever cases of diarrhea. Our results showed the prevalence of class 1 integrons is 31.4% among EPEC strains isolated from children with or without diarrhea. In all of EPEC isolates carrying class 1 integron, the *qacEr1* and *sull1* genes were identified. These genes determine resistance to antiseptics, disinfectants and sulfonamides, respectively (27).

The *aadA* genes were first described in 1985 (28). Some studies have shown the presence of *aadA* cassettes within class1 integrons that encode aminoglycoside 3'-9-adenylyltransferases that are responsible for streptomycin and spectinomycin resistance. Moreover, earlier study has shown that the *aadA* gene is highly conserved among STEC (29, 30).

Although streptomycin and spectinomycin have not been widely prescribed, *aadA* gene cassettes within integrons have been reported worldwide recently (12,

29). This confirms the fact that gene cassettes from the insert region of integrons are excised as covalently closed circles (31).

The cassettes with *dfrA* gene represented 100% of all EPEC strains that class 1 integrons found among them. *dfr* gene, which codes for dihydrofolate reductase, confer resistance to trimethoprim that it used largely to treat several infections caused by *E. coli* in Iran and other developing countries. Therefore these cassettes are able to become more prevalent. Previously, the role of these gene cassettes in resistance to trimethoprim have been mentioned clearly (12). *dfrA12*, *orfF*, and *aadA2* cassettes have become globally disseminated (30, 32). This cassette confers resistance to trimethoprim, streptomycin, and spectinomycin. Interestingly, these cassettes have detected in variety of Gram negative bacteria such as *E. coli*, *Salmonella* and *Serratia marcesens* (29, 32). Our present study showed high frequency of gene cassettes carrying *dfrA* gene among EPEC strains. In previous studies in Iran, the most prevalent gene cassettes among *Salmonella enteritidis* and *Pseudomonas aeruginosa* isolates were *aadA6-orfD* (33, 34).

It is important to focus on these findings that all of multiple drug resistance of EPEC strains in this study demonstrated the presence of integrons carrying antimicrobial resistance gene cassettes. Furthermore, these strains showed resistant to kanamycin and tetracycline that are prescribed to treat of drug resistant diarrhea. The combination of these findings shows an increasing problem in EPEC strains. Due to antibiotic selective pressures, these resistant EPEC strains could become predominant and colonize easily the gastrointestinal tracts of children. Low level of antibiotic resistance among EPEC strains in this study in compare to detected gene cassettes in class 1 integrons may be due to their gene expression.

Overall, these results indicate the widespread distribution of class 1 integrons containing gene cassettes conferring resistance to trimethoprim, streptomycin, and spectinomycin among EPEC strains. These gene cassettes are potentially capable of transmitting resistance determinants to other EPEC strains or to other types of *E.coli*. Moreover, bacterial strains bearing integrons might become resistant to third generation cephalosporins (35). This transmission of resistant is a serious threat to containment of infectious diseases caused by *E. coli*.

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