



The Prevalence of Typical and Atypical Enteropathogenic *Escherichia coli* Strains Isolated from Non-diarrheal Stool Samples of Children of Tehran

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Received: 27 August, 2024; Revised: 15 November, 2024; Accepted: 20 November, 2024

Abstract

Background: Diarrheagenic *Escherichia coli* (DEC) strains cause a self-limited gastrointestinal disease in developing countries and often infect children. Enteropathogenic *E. coli* (EPEC) is the most common DEC strain, which is usually carried by diarrheal children. However, some non-diarrheal EPEC carriers, particularly atypical enteropathogenic *E. coli* (aEPEC), have been increasing in some countries, including Iran.

Objectives: Our aim was to evaluate the prevalence of typical EPEC and atypical EPEC strains and their antimicrobial resistance rate among non-diarrheal children under 10 years of age, isolated from schools and welfare centers in Tehran.

Methods: We collected 350 fecal specimens from non-diarrheal children. The strains were analyzed for the presence of virulence genes such as *eae*, *bfpA*, *stx-1*, and *stx-2* genes. Antibiotic resistance rates of the strains were examined using the disk diffusion method.

Results: Among 350 fecal specimens, 160 specimens were taken from boys and 190 specimens from girls. The results of the genotypical-based assay for *eae*, *bfpA*, *stx-1*, and *stx-2* genes showed 32% of isolates were *eae* positive. Overall, of 142 isolates, 1.4% were only *bfpA* positive and 7.04% and 1.7% contained *stx-1* and *stx-2*, respectively. Resistance rate to tigecycline among EPEC strains was more reported than other antibiotics. On the other hand, 13.43% of isolates were considered Fosfomycin-susceptible EPEC strains.

Conclusions: The results showed the prevalence of atypical enteropathogenic *E. coli* strains is increasing in schools, and so there is a dangerous alarm as these carriers spread the antimicrobial-resistant strains in the community and can shift the microbial flora pattern.

Keywords: Enteropathogenic *Escherichia coli*, Non-diarrheal Children, Adhesion Genes

1. Background

Enteropathogenic *Escherichia coli* (EPEC), a gram-negative bacterium and opportunistic pathogen, is one of the most common members of the Enterobacteriaceae family. It causes various types of childhood gastrointestinal infections in developing countries (1-3). Often, EPEC-caused diarrhea is watery with abdominal cramps in children. Apart from the frequency of EPEC strains, these strains infect approximately 79,000 children worldwide every year. The infecting potential of EPEC, which is related to its

adhesion factors, is usually less than that of other bacteria causing gastrointestinal infections, although the frequency of EPEC is being reported more than other pathogens recently (4-6). Most studies have shown that the pathogenesis of EPEC strains is due to the formation of attaching and effacing lesions in host intestinal cells (7). These lesions can cause physiological changes in intestinal cells. Some of the virulence factors of EPEC strains involved in pathogenesis, such as *E. coli* attaching and effacing (*eae* gene) and bundle-forming pilus (*bfp* gene), were detected (8). These factors cause attachment and invasion of the strains into intestinal

cells. The *eae* and *bfp* genes are located on the enterocyte effacement pathogenicity island (PAI), locus of enterocyte effacement (LEE), and a plasmid called EPEC adherence factor (EAF), respectively (9, 10).

Enteropathogenic *E. coli* strains, based on the presence of the EAF plasmid, are characterized into two major groups: (1) Typical enteropathogenic *Escherichia coli* (tEPEC); and (2) atypical enteropathogenic *E. coli* (aEPEC) strains (11-13). While tEPEC strains have both *eae* and *bfp* genes (*eae*⁺ and *bfp*⁺), aEPEC strains do not have the *bfp* gene (*eae*⁺ and *bfp*⁻). Many studies have shown that aEPEC strains are more frequent than tEPEC strains in developing countries (10). The virulence gene encoding Shiga-like toxin (*stx* gene) causes bloody diarrhea by enterohemorrhagic *E. coli* (EHEC) strains. Studies have shown that the existence of *eae* without either of the *stx* alleles is a putative marker to confirm EPEC strains. The high prevalence of aEPEC strains has become a serious health issue among children.

2. Objectives

Our purpose was to study the prevalence of tEPEC and aEPEC strains and their antimicrobial resistance among non-diarrheal children under 10 years of age, isolated from schools and welfare centers in Tehran. The grouping of the children was performed according to sex and age groups.

3. Methods

3.1. Specimen Collection

A total of 350 non-diarrheal fecal specimens were collected from children under 10 years of age in schools and welfare centers of Tehran from October 2019 to March 2020. Certain criteria, including the existence of bacterial diarrheal disease (either bloody or watery diarrhea) in individuals for one month prior, individuals above 10 years of age, and antibiotic consumption within one week prior, were excluded from the study. These children were evaluated based on the history of diarrheal period, antibiotic use, and underlying disease. All of the children were categorized into three age groups: < 1 year, 1 to 5 years, and 5 to 9 years. Fecal specimens were collected in sterile containers and transferred to the laboratory for bacteriology experiments (14).

3.2. *Escherichia coli* Isolation

Initially, specimens were cultured on EMB agar and incubated at 37°C for 24 hours. Isolated colonies with a green metallic sheen and dark purple color were selected as *E. coli*. Phenotypic confirmation of *E. coli* isolates was done using the IMViC test, including SIM, TSI, MR-VP, and Simmons citrate agar as biochemical tests. To distinguish commensal *E. coli* strains from non-commensal strains, they were studied using a molecular test for *eae* and *bfpA* genes.

3.3. Genotypic Screening of Enteropathogenic *Escherichia coli* Strains

Escherichia coli isolates were investigated for the presence of the *eae* gene. As shown in Table 1, we used a pair of *eae* primers. The amplification of *eae* was described as follows: 94°C for 4 minutes for initial denaturation, followed by 30 cycles of 94°C for 33 seconds for denaturation, 64°C for 37 seconds for annealing, 72°C for 40 seconds for extension, and 72°C for 4 minutes as final extension. Grouping of the EPEC strains was done via amplification of *bfpA* among *eae*⁺ strains. The *bfpA* forward primer (5-AATGGTGCTTGCCTTGCTGC-3) and reverse primer (5-GCCGCTTTATCCAACCTGGTA-3) were used (15) and amplified as follows: 95°C for 4 minutes for initial denaturation, followed by 34 cycles of 94°C for 47 seconds for denaturation, 54°C for 38 seconds for annealing, 72°C for 55 seconds for extension, and 72°C for 6 minutes as final extension.

The control sample for both *eae* and *bfpA* genes was *E. coli* ATCC 2348/9. To ensure EPEC strains among *eae* positive strains, both *stx-1* and *stx-2* primers were used. Amplification of *stx-1* and *stx-2* was respectively performed as follows: 94°C for 9 minutes for initial denaturation, followed by 31 cycles of 94°C for 31 seconds for denaturation, 59°C for 32 seconds for annealing, 72°C for 30 seconds for extension, and 72°C for 3 minutes as final extension; 95°C for 6 minutes for initial denaturation, followed by 95°C for 31 seconds for denaturation, 63°C for 42 seconds for annealing, 72°C for 40 seconds for extension by 30 cycles, and 72°C for 7 minutes as final extension. *Escherichia coli* strain EDL 933 was selected as the positive control for both *stx-1* and *stx-2* genes. All PCR products were analyzed by electrophoresis with 1% agarose gel.

Table 1. PCR Primers Used for Detection of Enteropathogenic *Escherichia coli* in the Study

Primer	Sequence	Fragment Size (bp)	Reference
eae			
F	5-TGATAAGCTGCAGTCGAATCC-3	229	(16)
R	5-CTGAACCAGATCGTAACGGC-3		
bfpA			
F	5-AATGGTGCTTGCCTGCTGC-3	326	(15)
R	5-GCCGCTTTATCCAACCTGGTA-3		
stx-1			
F	5-GTGGCATTAACTGAATTGTCATCA-3	109	(17)
R	5-GCGTAATCCCACGGACTCTTC-3		
stx-2			
F	5-GGCACTGTCTGAACTGCTCC-3	255	(18)
R	5-TCGCCAGTTATCTGACATTCTG-3		

3.4. Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was performed by the disk diffusion method according to the clinical laboratory standards institute (CLSI) guidelines (19). The antimicrobial disks used in the study included meropenem (10 µg), ceftazidime (30 µg), cefotaxime (30 µg), fosfomycin (200 µg), co-trimoxazole (1/25 - 23/75 µg), tetracycline (30 µg), imipenem (10 µg), ciprofloxacin (5 µg), aztreonam (30 µg), amoxicillin-clavulanic acid (20/10 µg), and gentamicin (10 µg). *Escherichia coli* ATCC 25922 was used as the quality control. As described by the CLSI guidelines (19), all of the isolates were divided into three groups: (1) Sensitive, (2) intermediate resistance, and (3) resistant.

3.5. Statistical Analysis

Data from this study were statistically analyzed using SPSS software version 24. Chi-square and Phi and Cramer's V tests were performed, and $P < 0.05$ was statistically considered significant.

4. Results

4.1. Specimen Collection

Among the 350 fecal specimens collected, 160 specimens (45.71%) were from boys and 190 specimens (54.29%) from girls. The results showed that 45.71% of specimens were taken from children above 5 years of age, 37.42% were from children between 1 to 5 years of age, and 16.85% were from children under 1 year of age.

4.2. Enteropathogenic *Escherichia coli* Strains Screening

All 350 fecal samples, whose biochemical characteristics were evaluated, were described as *E. coli*. The next step was to identify EPEC strains using a PCR-based assay. The results of PCR for *eae*, *bfpA*, *stx-1*, and *stx-2* genes showed 32% of isolates were *eae* positive (see Figure 1), and other genes were negative. On the other hand, among the 142 *eae*-positive strains, 89.43% were *bfpA*-negative strains and only 1.4% were *bfpA* positive (see Figure 2), which were typical enteropathogenic *E. coli* and atypical enteropathogenic *E. coli* strains, respectively (Table 2).

4.3. Antimicrobial Susceptibility Testing

The study on antimicrobial resistance of aEPEC strains was performed based on the sex and age group of children (see Table 3). Among the antibiotics studied, amoxicillin-clavulanate-resistant *E. coli* isolates were significantly reported (P -value = 0.03). The highest resistance rate to tetracycline was reported (25% in 5 to 9 years, 28.75% in 1 to 5 years, and 22.22% in < 1-year-old children). On the other hand, the least resistance rate was observed for imipenem (0% in both boys and girls), meropenem (0% in both boys and girls), azithromycin (5% in boys and 2% in girls), and ciprofloxacin (2.38% in boys and 2% in girls).

5. Discussion

Enteropathogenic *E. coli* is the second most frequent pathotype causing traveler's diarrhea in most developing countries (20). Enteropathogenic *E. coli* strains are not detected with routine protocols of stool culture in clinical laboratories; however, with the use of molecular techniques, EPEC strains have been identified recently (21). Almost all enteropathogenic *E. coli* strains cause symptomatic diarrhea and have become an important public health issue for children (22). Despite the decrease in the incidence of gastrointestinal infections caused by these strains in developed countries, EPEC strains are increasing in developing countries. On the other hand, the high rate of antibiotic resistance in these strains has generally become a major therapeutic challenge (23). Additionally, there is an increasing health issue in developing communities. Recently, several cases of EPEC colonization in asymptomatic children have been observed (24).

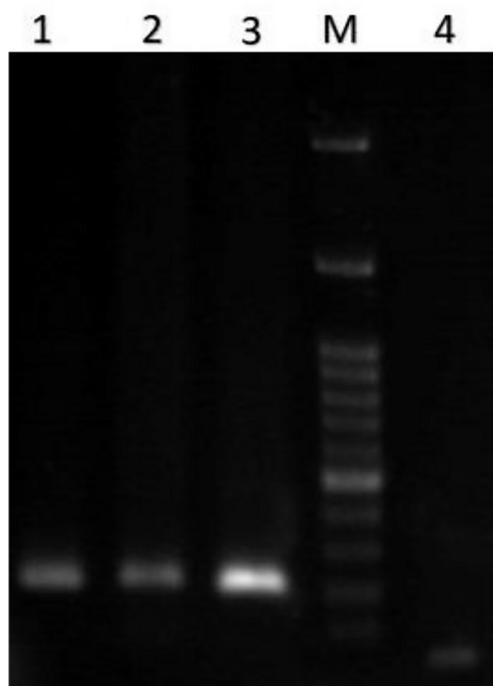


Figure 1. PCR productions of *eae* gene of enteropathogenic *Escherichia coli* (EPEC) strains (229 bp). Lane 1 and 2, clinical isolates; lane 3, positive control; lane M, DNA marker (100 bp-1 kbp); lane 4, negative control

Asymptomatic colonization could increase the prevalence of the strains and even antimicrobial-resistant strains. In the current study, the frequency of aEPEC strains was evaluated among non-diarrheal children. The results of the study showed that the prevalence of asymptomatic colonization of EPEC among children under 10 years was higher. While Chellapandi et al. reported 12 of 50 EPEC strains in children (16), the results of Darbandi et al. (2012) showed that from 158 *E. coli* isolates, 50 strains were isolated (25). Asymptomatic colonization is much more common in children, with our study showing the most frequent age group for EPEC colonization was reported between 4 to 9 years. In several similar studies, these strains were more frequently reported in ≤ 5 -year-old children (26-28). According to the results, prevalence in these age groups is more reported due to the presence of children in public places such as schools and also weak health surveillance in communities (29).

Female patients are much more prevalent than males. Afset et al. reported that the prevalence of aEPEC

among female carriers (40.6%) is higher than in males (10). Similarly, our data indicated that girls showed more prevalence than boys. Based on studies, all EPEC strains have the *eae* gene, and EPEC strains without the *bfpA* gene are considered aEPEC strains. We differentially studied the frequency of each of the tEPEC and aEPEC strains. Atypical strains are more frequent than typical strains in children. Atypical strains have become important gastrointestinal *E. coli* pathotypes, with their frequency increasing. Snehaa et al. found 2.5% typical isolates and 7% atypical isolates among 19 EPEC (30). Similarly, our findings demonstrated that these pathotypes are more frequent than typical strains. The results of the study on *eae*-positive strains showed that most strains did not have the *bfpA* gene, and only in 3 of the *eae*-positive strains was the *bfpA* gene detected. Darbandi et al. similarly reported the *bfpA* gene (0.7%) among EPEC strains (25).

The higher frequency of aEPEC strains is due to the fact that these strains usually cause non-diarrheal disease, and asymptomatic carriers can easily spread

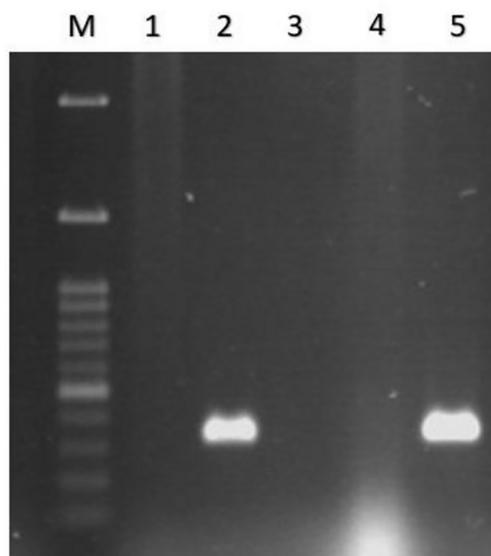


Figure 2. PCR products of *hfpA* gene of enteropathogenic *Escherichia coli* (EPEC) strains (324 bp). Lane M, DNA marker (100 bp, 1000 bp); lane 1, clinical isolate; lane 2, positive control; lane 3, negative control; lanes 4 and 5, clinical isolates

Table 2. Frequency of Enteropathogenic and Enterohemorrhagic *Escherichia coli* Virulence Genes Studied Among Children Less Than 10 Years of Age^a

Virulence Genes	<i>eae</i>		<i>hfpA</i>		<i>stx-1</i>		<i>stx-2</i>		P-Value ^b	CI ^c
	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative		
Age (y)									0.542	0.534 - 0.550
<1	16 (14.28)	43 (12.28)	0 (0)	59 (54.12)	1 (10)	58 (17.05)	0 (0)	59 (16.95)		
1-5	43 (38.39)	88 (25.14)	3 (100)	53 (48.62)	3 (30)	128 (37.64)	2 (100)	129 (37.06)		
5-9	53 (15.14)	107 (30.57)	0 (0)	67 (61.46)	6 (60)	154 (45.29)	0 (0)	160 (45.97)		
Total	112	238	3	109	10	340	2	348		

^a Values are presented as No. (%) unless otherwise indicated.

^b P < 0.05 is significant.

^c Confidence interval was computed to 95%.

them in the community, leading to a higher frequency compared to tEPEC. In recent years, extensive antibiotic consumption has produced antibiotic-resistant *E. coli* isolates. Canizalez-Roman et al. showed that EPEC is one of the most prevalent antibiotic-resistant pathotypes (31).

Evaluation of the antibiotic resistance rate in our study exhibited that EPEC strains had higher resistance to amoxicillin-clavulanate, tetracycline, and trimethoprim-sulfamethoxazole than other antibiotics, while among the antibiotics studied, imipenem and

gentamicin were reported to have the highest effectiveness on strains. The present study was epidemiologically performed over a limited period and did not study several factors such as the expression of adhesion factors and resistance genes, i.e., beta-lactamase genes. The limitation of this study was that it did not perform more comprehensive antimicrobial resistance tests, both genotypical and phenotypical, and finally did not identify multi-drug resistant strains such as ESBLs and carbapenem-resistant strains, because the aim was to study the epidemiological prevalence of non-diarrheal children infected with enteropathogenic *E.*

Table 3. Antimicrobial Susceptibility Testing of Enterohemorrhagic *Escherichia coli* Strains Isolated from Children Less Than 10 Years of Age by Gender

Variables	Age (Boy) ^a			Age (Girl) ^a			P-Value ^b			
	<1	1-5	5-9	<1	1-5	5-9	Age (Boy)	CI ^c	Age (Girl)	CI ^c
SXT							0.735	0.727 - 0.744	0.585	0.575 - 0.594
S	0	2.38	2.38	0	1.21	2.54				
I	0	0	0	0	0	0.84				
R	22.22	25	22.61	21.95	21.95	22.88				
AMC							0.038	0.034 - 0.042	0.030	0.026 - 0.033
S	3.70	0	0	2.43	0	0				
I	7.40	1.19	0	2.43	1.21	5.93				
R	22.22	26.19	25	24.3	23.17	21.18				
AZT							0.821	0.813 - 0.828	1.0	1.0
S	29.62	23.80	21.42	21.95	23.17	26.17				
I	0	1.19	2.38	0	0	0				
R	0	2.38	2.38	0	1.21	0.84				
CAZ							0.77	0.768 - 0.784	0.74	0.737 - 0.754
S	18.51	20.23	20.23	21.95	23.17	23.72				
I	0	2.38	2.38	0	0	0.84				
R	7.40	6	2.38	0	1.21	0.84				
CP							0.462	0.452 - 0.472	1	1
S	18.51	27.3	21.42	21.95	23.17	26.27				
I	3.70	0	2.38	0	0	0				
R	0	1.19	1.19	0	1.21	0.84				
CTX							0.551	0.541 - 0.560	1.0	1.0
S	29.62	23.80	21.42	21.95	23.17	25.42				
I	3.70	2.38	0	0	0	0				
R	3.70	2.38	3.57	0	1.21	0.84				
FOS							0.227	0.219 - 0.236	0.406	0.396 - 0.416
S	3.70	1.19	3.57	0	2.43	2.54				
I	14.81	22.61	20.23	21.95	20.17	21.18				
R	7.40	4.76	1.19	2.43	0	2.54				
GM							0.448	0.438	0.740	0.731 - 0.748
S	22.22	20.23	20.23	21.95	20.17	22.03				
I	0	6	2.38	2.43	2.43	5.08				
R	0	2.38	2.3	0	0	0				
IPM							0.607	0.598 - 0.617	-	-
S	22.22	26.19	25	21.95	25	25.42				
I	0	2.38	0	0	0	0				
R	0	0	0	0	0	0				
MEM							0.098	0.092 - 0.104	0.156	0.148 - 0.163
S	18.51	23.80	25	21.95	25	25.42				
I	3.70	4.76	0	2.43	0	0				
R	0	0	0	0	0	0				
TE							0.125	0.119 - 0.132	0.434	0.424 - 0.443
S	3.70	0	0	0	1.21	0				
I	0	0	0	0	0	0.84				
R	22.22	28.57	25	21.95	23.17	25.42				

Abbreviations: SXT, trimethoprim-sulfamethoxazole; AMC, amoxicillin-clavulanate; AZT, azithromycin; CAZ, ceftazidime; CTX, cefotaxime; FOS, Fosfomycin, gentamicin; IPM, imipenem; MEM, meropenem; TE, tetracycline; S, sensitive; I, intermediate; R, resistant.

^a Values are presented as %.

^b P < 0.05 is significant.

^c Confidence interval was computed to 95%.

coli. Therefore, we recommend that more factors should be investigated.

5.1. Conclusions

The results of our study indicate that asymptomatic carriers of EPEC strains, as well as their antimicrobial

resistance rates, are increasing in schools. This is a dangerous alarm because these carriers spread the antimicrobial-resistant strains in the community and can shift the microbial flora pattern.

Acknowledgements

We thank the staff of preschools and welfare centers in Tehran for their assistance, which helped us perform the study more effectively.

Footnotes

Authors' Contribution: S. F. performed as the original experimental worker. S. N. P. designed the basis of the study. B. B. served as a co-writer of the manuscript and was recruited as the responsible co-supervisor of the study. K. E. F. B. revised the manuscript and also re-analyzed the statistical data.

Conflict of Interests Statement: The authors declared that they have no conflict of interest.

Data Availability: No new data were created or analyzed in this study. Therefore, data sharing does not apply to this article.

Ethical Approval: All procedures of the study were approved by the Ethics Committee of Tarbiat Modares University ([IR.MODARES.REC.1398.033](https://doi.org/10.1016/j.trstmh.2008.03.017)).

Funding/Support: This study was funded by the Tarbiat Modares University Fund.

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