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Investigation of enteropathogenic *Escherichia coli* and Shiga toxin-producing *Escherichia coli* associated with hemolytic uremic syndrome in İzmir Province, Turkey

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Background/aim: The purpose of this study was to investigate Shiga toxin-producing *Escherichia coli* (STEC) and enteropathogenic *Escherichia coli* (EPEC) strains originating from diarrheagenic patients.

Materials and methods: A total of 102 patients with diarrhea between October 2012 and January 2013 were enrolled in this study. Multiplex and standard polymerase chain reactions were performed to detect and distinguish STEC and EPEC strains. O serotyping of EPEC was carried out by monovalent antisera. The O and H serotyping of STEC strains was performed at the Refik Saydam Institute, Ankara.

Results: A total of 5 (3.42%) strains were identified as STEC, and 3 strains (2.05%) were atypical EPEC. One of the STEC serotypes was O157:H7 carrying *VT1*, *Stx1A*, and *escv* genes. The other STEC strain was identified as O174:H21, which is associated with hemolytic uremic syndrome and consists of *VT2* and *Stx2A* genes. One of the EPEC and three of the STEC serotypes were nontypeable. The serotypes of the atypical EPEC strains were identified as O114 and O26.

Conclusion: To the best of our knowledge, this is the first report of O174:H21 from the İzmir region that was shown to be a Shiga toxin-producing non-O157 serotype of STEC.

Key words: Enteropathogenic *Escherichia coli*, Shiga toxin-producing *Escherichia coli*, diarrhea, polymerase chain reaction, serotyping, MALDI-TOF mass spectrometry

1. Introduction

Pathogenic *Escherichia coli* strains are one of the most important bacteria underlying diarrhea in children and adults. Pathogenic *E. coli* strains are classified according to their virulence factors as follows: Shiga toxin-producing *E. coli* (STEC), enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), and diffuse adherence *E. coli* (DAEC) (1,2).

EPEC have been associated with infant diarrhea in the developing world (3–5); infections with EPEC strains were significantly more common in children (6). According to the Global Enteric Multicenter Study, which was a population-based case control study, typical EPEC strains cause diarrhea for ≤4 years in African and Asian children (7). Additionally, atypical EPEC strains were reported as the predominant cause of gastroenteritis in the water supply of Melbourne, Australia (2). In recent years, very few studies have reported atypical EPEC strains from patients with diarrhea in Turkey (8,9). EPEC strains are defined as intimin-containing diarrheagenic *E. coli* not producing Shiga toxin. The main characteristic of EPEC strains is that they can create attaching-effacing lesions in the intestine. The genes required for the production of these lesions are located on a pathogenicity island known as the locus for enterocyte effacement (LEE) (10).

The EPEC also can include chromosomal gene *eae* (1). The *eae* and *escv* genes of the EPEC strains encode virulence factors responsible for the attaching and effacing lesions. However, *bfp* is a structural gene that is named for a bundle-forming pilus encoded by the 90-kb *pEAF* and is specific only for the EPEC strains (11). EPEC strains are divided into two groups, typical and atypical EPEC, which are further classified into the *escv, bfp*, or *eae* genes. Typical EPEC strains (tEPEC) carry the *escv* or *eae* and *bfp* gene. However, atypical EPEC (aEPEC) consist of only the *escv or eae* genes without the *bfp* gene (1,12).

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STEC are also defined as an enterohemorrhagic *Escherichia coli* (EHEC) or verocytotoxigenic *Escherichia coli* (VTEC) (13). They have common virulence properties and they lead to a number of human gastrointestinal diseases such as diarrhea, bloody diarrhea, hemolytic uremic syndrome (HUS), and hemorrhagic colitis (HC). One of the most important serotypes of STEC is O157:H, and STEC strains produce Shiga toxins (Stx) 1 and/or 2 encoded by the *stx1A* and *stx2A* genes (11). The main reservoirs of STEC strains are food and animals, which harbor these bacteria in their intestinal tract systems. These bacteria can also spread to humans via fecally contaminated food and/or water (14). STEC are mainly from butchering environments, vegetable samples, and fecal samples of children and animals (15–20). *E. coli* pathotypes are a health problem for the public, and diarrheagenic *E. coli* strains might not be detected efficiently by cultural and biochemical methods. Therefore, identification of these strains is problematic in a variety of clinical laboratories. Although many studies have been performed on the pathogenesis of EPEC strains, there is no satisfying information about the clinical diagnosis of EPEC infection associated with diarrhea. Traditional methods to investigate diarrheagenic *E. coli* strains are laborious and nonsuitable for daily clinical use. Furthermore, discrimination of EPEC from STEC strains can provide more accurate diagnosis and proper clinical treatment. Therefore, we aimed to investigate and discriminate STEC and EPEC strains from infant and adult diarrhea samples. To achieve that goal we compared standard and multiplex polymerase chain reaction (PCR) efficiency and amplified Shiga toxigenic genes (*VT1*, *VT2*, *Stx1A*, and *Stx2A*), the bundle-forming pilus gene (*bfpB*), and the genes for virulence factors responsible for the attaching and effacing lesions (*eae* and *escv*).

2. Materials and methods

2.1. Clinical specimens

There were 102 diarrhea patients (62 children and 40 adults) whose stool samples were examined for EPEC and STEC strains between October 2012 and January 2013. The stool samples were selected from the patients who had symptoms including three or more loose, liquid, or watery stools within a 24-h period. The presence of leukocytes and/or erythrocytes by microscopic examination was the selection criterion of the stool samples. The diagnosis of viral gastroenteritis was excluded. Stool samples were also screened for *Salmonella* spp., *Campylobacter* spp., and *Shigella* spp. The distribution of the hospital wards and the number of patients associated with diarrhea are shown in Figure 1.

2.2. Isolation and identification of *E. coli* strains

Stool samples were transported immediately to the laboratory and cultured directly on Sorbitol MacConkey medium (SMAC) (Sigma), MacConkey medium (MAC) (Sigma) and Fluorocult®*E. coli* O157:H7 culture Medium (Merck). The colonies that were sorbitol-negative or were positive on SMAC and Fluorocult®*E. coli* O157:H7 medium and were lactose-positive on MAC were selected following overnight incubation at 37 °C (Table 1). Colonies with a metallic green sheen were selected followed by culturing on eosin methylene blue agar (EMB) plates (Merck) for further identification. The *E. coli* strains were confirmed by IMVIC tests and MALDI-TOF mass spectrometry (BioMérieux, France), respectively.

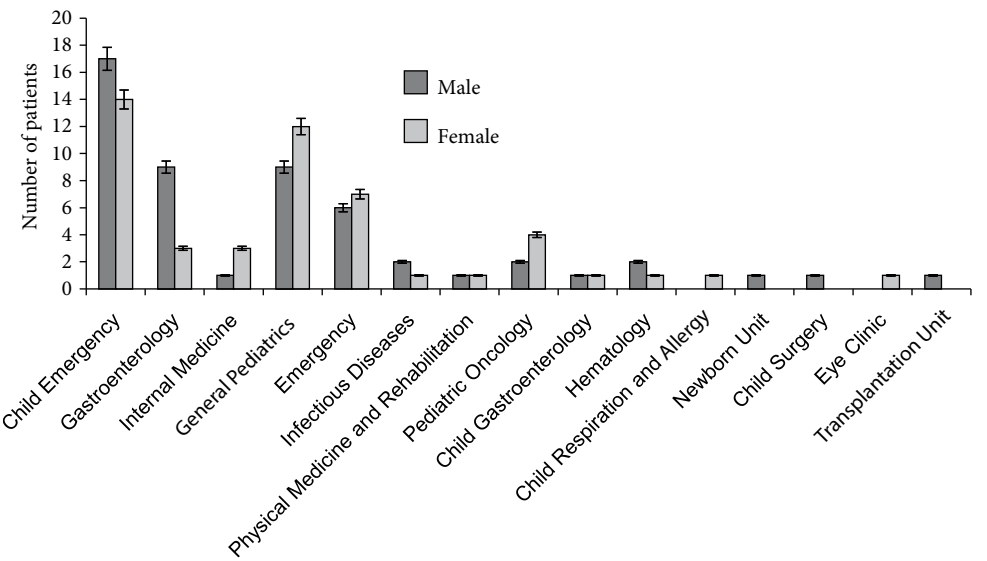

Figure 1. The distribution of the hospital wards and the number of patients with diarrhea.

Table 1. Results of culture media reactions of EPEC and STEC strains.

Isolate Code	Serotype	Culture media			
		SMAC sorbitol (+ / -)	MAC lactose (+ / -)	Fluorocult®*E. coli* O157:H7 sorbitol (+ / -)	EMB metallic green sheen (+ / -)
D-14-1	O157:H7	-	+	-	+
D-73-2*	O174:H21*	+	+	+	+
D-40-1	NT[a]	+	+	+	+
D-41-3	O114	-	+	-	+
D-13-1	O26	-	+	-	+
D-35-1	NT[a]	-	+	-	+
D-68-3	NT[a]	-	+	-	+
D-89-3	NT[a]	-	+	-	+
E. coli O157:H7 positive control	O157:H7	-	+	-	+ +

a: Nontypeable. *: Shiga toxin-producing *E. coli*; sorbitol (+).

2.3. Multiplex and standard PCR to detect STEC and EPEC

Genomic DNA isolation was performed using the PureLink® Genomic DNA Isolation Kit (Invitrogen). Standard and multiplex PCR was performed as described in the literature (12,21) to detect EPEC and STEC strains. The genes *VT1* (348 bp), *VT2* (584 bp), and *eae* (863 bp) were measured by standard PCR, and the genes *escv* (534 bp), *bfpB* (826 bp), *Stx1A* (250 bp), and *Stx2A* (325 bp) were targeted for multiplex PCR analysis.

The multiplex PCR method was slightly modified (12) as follows: the multiplex PCR was performed in a 25-µL reaction mixture consisting of 1.25 U of Taq DNA polymerase (Fermentas, Germany), 1X Taq polymerase buffer (Fermentas), 0.3 mM concentration of each dNTP (Fermentas), and 0.4 mM of each primer (Sentromer, Turkey). Thermal cycling conditions were as follows: 95 °C for 5 min for the initial denaturation, 30 cycles of 95 °C for 1 min/58 °C for 40 s/72 °C for 1 min, and a final extension at 72 °C for 5 min. The PCR products were run on 1% agarose and were visualized with a gel Doc system (UVP® 97-0192-01 MultiDoc-It™ UV Imaging System with M-20, USA).

Another notable thing is that the STEC strains were characterized as expressions of toxins with variations in the amino acid sequence. These variants are grouped within the Stx1 and Stx2 types. Therefore, we used other primer sequences, including *VT1* (verocytotoxin type 1) that causes systemic symptoms, the *eae* gene that is essential for the strains to attach to the host mucosal surface, and *VT2* that is the subtype of VT divided into *VT1*, *VT2*, *VT2c*, and *VT2e*. These are functionally the same within the range of STEC; the *eae* gene can be found in STEC. Thus, standard PCR was performed (21) with optimization of the annealing temperature at 64 °C for the VT1 and VT2 primers (BM Laboratory Systems, Turkey). These validated the *stx1A* and *stx2A* genes.

E. coli O157:H7 (RSKK 234) (Refik Saydam Institute Culture Collection, Ankara, Turkey) was used as a positive control strain for the *bfp*, *eae*, *escv*, and *stx1A* as well as *stx2A*, *VT1*, and *VT2* genes for the multiplex and standard PCR, respectively.

2.4. Serotyping

O serotyping of EPEC was performed according to monovalent antisera used for serological identification of EPEC cultures via the slide agglutination method against the O antigen (O124, O26, O55, O86, O111, O119, O125, O126, O127, O128, O114, and O142) (Bio-Rad, France) (22). A positive reaction corresponds to the appearance of massive and immediate agglutination as well as the conformation of EPEC. The O and H serotyping of STEC strains was performed at the Refik Saydam Institute in Ankara, Turkey.

3. Results

3.1. Characteristic of subjects

A total of 102 adult and pediatric stool samples from various hospital wards were examined to detect STEC and EPEC strains. Of the 102 patients, 62 (60.78%) were children and 40 (39.21%) were adults (Figure 1).

3.2. Bacterial strains and multiplex and standard PCR

We identified 146 *E. coli* strains via cultural and biochemical tests including IMVIC and MALDI-TOF®MS (BioMérieux, France). Of the 146 *E. coli* strains, 5 were STEC (3.42%) and 3 were atypical EPEC (2.05%) strains;

the other 138 strains of *E. coli* (94.52%) were reported according to the MALDI-TOF®MS data library.

One of the STEC strain was identified as an atypical non-O157 STEC serotype, which is O174:H21 and is associated with HUS (Table 2). The other STEC strain was serotyped as O157:H7. The *eae* gene was positive for the EPEC strains, also called atypical EPEC. The Shiga toxigenic genes were positive for the STEC strains. The distribution of the *escv*, *eae*, *VT1*, *VT2*, *Stx1A*, and *stx2A* genes of the STEC and EPEC are shown in Figures 2–4. The STEC strains were sorbitol-negative except for the O174:H21 STEC strain that was sorbitol-positive. The EPEC strains were sorbitol-negative on SMAC and Fluorocult®*E. coli* O157:H7 culture media (Table 1).

While screening the stool samples in accordance with the presence of *Salmonella* spp., *Campylobacter* spp., and *Shigella* spp., we found that isolate D-40-1 (an atypical EPEC) was coinfected with *Campylobacter jejuni* in a pediatric emergency patient. Additionally, 3 patients that originated from the emergency service were diagnosed with *C. jejuni*, *Shigella sonnei*, and *Salmonella enteridis*; these were characterized by a large number of leukocytes via microscopic examination.

3.3. Serotyping

Serotyping of the STEC strains was performed at the Refik Saydam Institute in Ankara, Turkey. Serotypes of the STEC strains were determined to be O157:H7 and an untypical serotype O174:H21. However, 3 of the STEC strains were not designated serologically. The EPEC strains were typed as O114 and O26 by the slide agglutination method. The serotypes of EPEC and STEC strains are shown in Table 2.

4. Discussion

Diarrhea is one of the major reasons for morbidity and mortality in the developing world. This situation receives relatively little attention in industrialized countries (23). Pathogenic *E. coli* strains are one of the most significant pathogens that lead to diarrhea. However, nonpathogenic strains of *E. coli* are normally localized in the intestinal tract of humans and other warm-blooded animals (23). The EPEC and STEC strains are pathotypes of diarrheagenic *E. coli* strains and are associated with their O (lipopolysaccharide) and H (flagella) antigens.

To initially identify the pathogenic EPEC and STEC strains via culture methods, sorbitol fermentation was used to discriminate between pathogenic and nonpathogenic strains of *E. coli*. The O157 serotypes of STEC are occasionally sorbitol-negative (24,25). However, sorbitol-fermenting O157 strains have begun to be reported (26–29). In our study, we reported that the *E.coli* O174:H21 strain (n = 1) that fermented sorbitol was isolated from the pediatrics service, but other STEC strains (n = 4) were not sorbitol-fermenting. Detection of pathogenic *E. coli* strains, especially EPEC and STEC, requires that the sorbitol-fermenting strains be considered to avoid missing pathogenic strains on culture media including sorbitol. This might be helpful for accurate diagnosis of diarrhea patients.

STEC strains are critically important pathogens because of their association with HUS and HC (30). *E. coli* O157:H7 can produce two different Stx: Shiga-like toxin 1 (Stx1) and Shiga-like toxin 2 (Stx2). Stx1 is very similar to the Shiga toxin of *Shigella dysenteriae*, and Stx2

Table 2. Characterization of EPEC and STEC strains.

Virulence genes of EPEC and STEC									
Isolate code	Symptoms	*VT1*	*VT2*	*eae*	*Stx1A*	*Stx2A*	*escv*	Serotype	Department/service
D-14-1	Diarrhea[b]	+	-	-	+	-	+	0157:H7	General pediatrics
D-73-2	Diarrhea[b]	-	+	-	-	+	-	O174:H21	General pediatrics
D-40-1	Diarrhea[b]	-	-	+	-	-		N.T[a]	Child emergency
D-41-3	Diarrhea[b]	-	-	+	-	-	-	O114	General pediatrics
D-13-1	Diarrhea[c]	-	-	+	-	-	-	O26	Child emergency
D-35-1	Diarrhea[b]	-	-	-	+	-	+	N.T.[a]	Hematology
D-68-3	Diarrhea[c]	-	-	-	+	-	-	N.T.[a]	Emergency
D-89-3	Diarrhea[b]	-	-	-	+	-	-	N.T.[a]	Child oncology
E. coli O157:H7 (RSKK 234)	-	+	-	+	+	-	+	O157:H7	-

a: Nontypeable. b: Diarrhea with leukocytes and erythrocytes. c: Diarrhea with leukocytes.

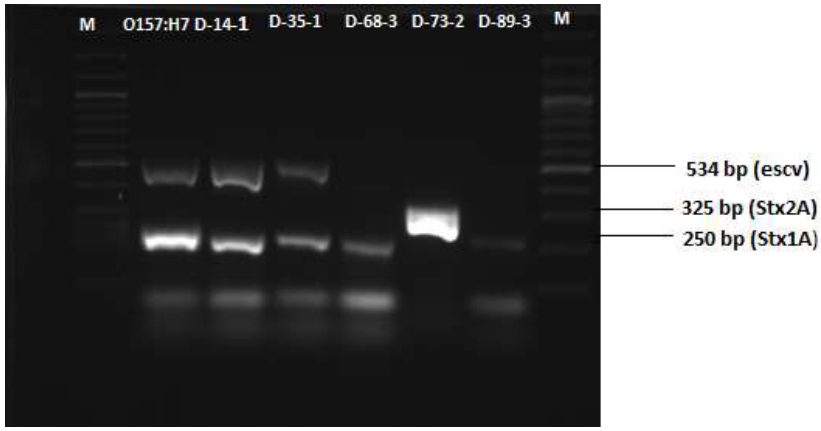

Figure 2. Multiplex PCR for *escv*, *stx1A*, and *stx2A*. M: Ladder, 100 bp (Fermentas, Germany), *E. coli* O157:H7; positive control, D-14-1, D-35-1, D-68-3, D-73-2, and D-89-3.

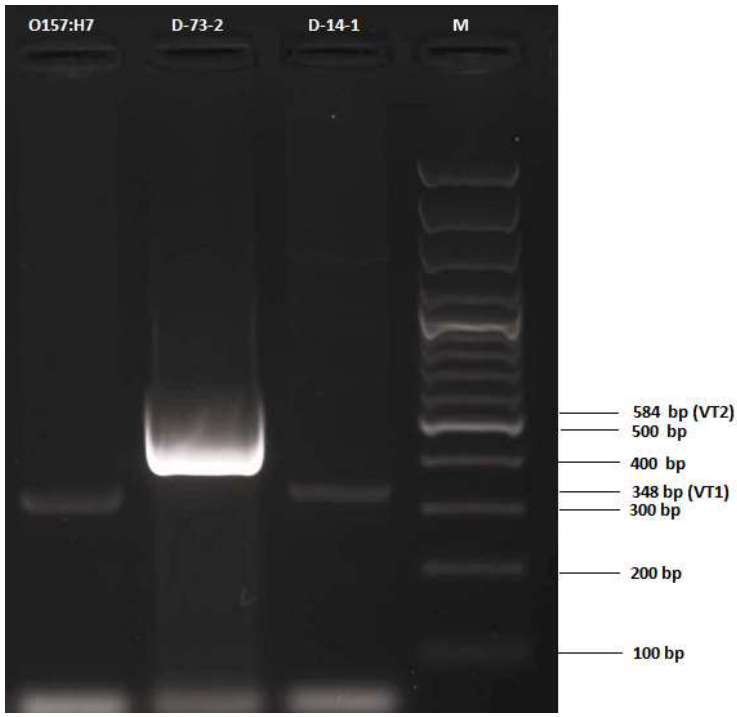

Figure 3. Standard PCR for *VT1* and *VT2*: *E. coli* O157:H7; positive control, D-73-2, D-14-1, M: Ladder: 100 bp (Fermentas, Germany).

is genetically different from Stx1 (31). During 2007–2010, the most commonly reported serotype was O157:H7 (774 out of 2140 fully serotyped cases), followed by O157:H- and O103:H2. In 2011, the most commonly reported serotype was O104:H4 (118 out of 686 fully serotyped isolates), followed by O157:H- and O157:H7 (32).

In our research, we found 146 strains of *E. coli*, including 5 STEC strains (3.42%). One STEC serotype was O157:H7, and the other was O174:H21. However, three STEC strains were nontypeable. Keskimaki et al. reported 8 STEC out of 603 diarrhea patients in Finland (22). Similarly, Kalantar et al. reported 5.7% Shiga toxin-producing *E. coli* originating from children with acute diarrhea in Iran (33). STEC strains are also considered foodborne pathogens that may cause human diseases (34). In Turkey, STEC strains are found occasionally. These

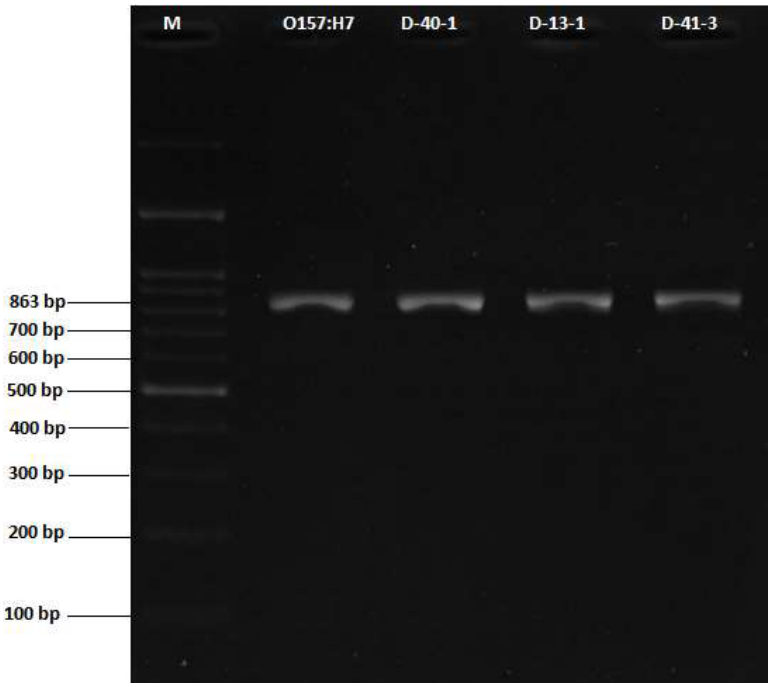

Figure 4. Standard PCR for *eae* gene: M: Ladder; 100 bp (Fermentas, Germany), *E. coli* O157:H7; positive control, D-40-1, D-13-1, and D-41-3.

come from food products, cattle carcasses, and Anatolian water buffaloes (35–37). However, few studies have been performed directly on the diarrhea from patients.

Yeniiz et al. detected five O157:H7 STEC serotypes from 429 cases (38). Erdogan et al. reported one O157:H7 serotype in Ankara as a case report (13). We also detected one O157:H7 serotype from STEC in a pediatric patient with diarrhea. As we understand the literature, screening of the O157:H7 serotype is not efficiently reported in HUS patients. Nevertheless, because the O157:H7 serotype is one of the most significant foodborne pathogens, researchers might mainly focus on the transmission source of the O157:H7 serotype in Turkey.

The *E. coli* O174:H21 strains are non-O157:H7 STEC and are variably found in stool from HUS patients. One of our patients had HUS complications with diarrhea and was treated with liquid and diet support; culture from the stool was positive for *E. coli*, and molecular detection of the Shiga toxin gene (*stx2A*) confirmed it as STEC by PCR. The serotype was O174:H21 (Figure 3). A number of Shiga toxigenic but non-O157:H7 *E. coli* strains have been reported including O26:H11, O91:H21, O111:H-, O145:H-, and O174:H21 (39,40). Similarly, we reported an uncommon Shiga toxin producing a non-O157 serotype for the first time in Turkey. The strain O174:H21 was derived from a 15-year-old male patient with abdominal pain and vomiting for 1 week. Ileus was prediagnosed, and the patient was transmitted to the pediatric surgical clinic for therapy with a supplemented liquid diet.

The serotypes of the EPEC strains were detected as O114 and O26 (Tables 2 and 3). The serotype of O26 for the EPEC strains was also determined for the STEC strains. These were isolated from the HUS patients because both STEC and EPEC strains of the O26 serotype share the locus for enterocyte effacement (41). The EPEC strains were named from the point of their negative characteristics, particularly their inability to produce enterotoxins or to designate *Shigella*-like invasiveness. The EPEC strains

Table 3. The distribution of STEC, EPEC, and other strains of *Escherichia coli*.

	STEC (n/%)	EPEC (n/%)	Other strains of *E. coli* (n/%)
E. coli	5 (3.42)	3 (2.05)	138 (94.52)
Total	5	3	138

were divided into two different categories, i.e. typical and atypical, contingent upon the presence or absence of the EPEC adherence factor (EAF) plasmid. There were 12 serotypes including O26, O55, O86, O111, O114, O119, O125, O126, O127, O128, O142, and O158 (42,43).

The epidemiological importance of EPEC strains remains unclear. However, various studies have already reported the significance of the EPEC strains. For example, Hien et al. carried out a case control study in Vietnam with children aged <5 years with diarrhea and reported 7 EPEC out of 249 children (44). Similarly, Aslani and Alikhani reported 36 EPEC strains from children with diarrhea and without diarrhea and 14 atypical EPEC strains identified from healthy children in Iran (10). In our study, we detected 3 EPEC strains from children with diarrhea symptoms including leukocytes and erythrocytes in the microscopic examination. These patients were from the pediatric emergency service and general pediatrics clinics. Souza et al. reported 1 atypical EPEC out of 515 *E. coli* strains from children in Brazil (45). Turhanoglu et al. reported 153 (60.9%) EPEC strains out of 1079 children suffering from diarrhea in Southeast Anatolia. Aydın Tutak and Tuğrul reported one atypical EPEC strain including the *eae* gene in İstanbul (8). However, there is no serotype information on these EPEC strains. Similarly, we reported 3 EPEC strains including those that carried only the *eae* gene without *bfpB*. To the best of our knowledge, this is the first report of the O114 and O26 EPEC serotypes from the İzmir region of Turkey. The O114 and O26 serotypes were positive for the *eae* genes.

Our study shows that culture-based and PCR methods can discriminate between STEC and EPEC strains. However, they cannot always be used to predict treatment response. To discriminate between STEC and EPEC strains, PCR methods should be optimized for correct and rapid identification. This is a limitation of this study.

It is significant that we detected pathogenic *E. coli* except for the *E. coli* strains that are members of the normal intestinal flora. Data about the incidence and prevalence of the EPEC and STEC strains in Turkey are limited, and diagnosis of these strains should be regarded as an important contribution to therapy. In our study, we showed that *E. coli* induces diarrhea in adults and infant patients. Our data indicates that *E. coli* O157:H7 is a primary concern in public health; however, other pathogenic pathotypes of *E. coli* have started to draw attention. These include *E. coli* O174:H21, which is related to HUS and HC in children, as well as strains EPEC O26 and EPEC O114 in Turkey.

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