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RESEARCH ARTICLE

Research into the Presence of Shiga Toxin-producing Escherichia coli in Human and Cattle Feces with Culture, ELISA and Molecular Methods

Selahattin ÜNLÜ[1*], Hamdi Murat TUĞRUL[2]

[1]*Department of Medical Microbiology, Faculty of Medicine, Afyonkarahisar Health Sciences University, Afyonkarahisar, Türkiye*
[2]*Department of Medical Microbiology, Faculty of Medicine, Trakya University, Edirne, Türkiye*

ABSTRACT

According to 2021 European Food Safety Authority (EFSA) data, Shiga-toxin (stx) producing *Escherichia coli* (STEC) is the fourth most frequently observed zoonotic agent in humans after *Campylobacter, Salmonella* and *Yersinia*. It may cause very serious infections like hemolytic uremic syndrome (HUS) and hemorrhagic colitis (HC). In 2011 it caused a large epidemic, leading to the death of 54 people and the development of HUS in several people in Germany. For diagnosis, the detection of STEC bacteria is an important marker to indicate the formation of the toxin. In this study, the aim was to determine the frequency of STEC in patients referred for fecal cultures and to identify the colonization rates of this microorganism among cattle in an integrated meat facility. A total of 250 human fecal samples and fecal samples from 180 cattle collected from an integrated meat facility were investigated for the presence of STEC. None of the samples from humans had STEC identified. Of the animal samples, 11 were positive with enzyme linked immunosorbent assay (ELISA). Of the samples positive with ELISA, 8 were also positive with polymerase chain reaction (PCR). Of the samples positive with PCR, 3 could proliferate on CROMagar STEC medium. Of the 3 isolated bacteria, 1 was serotyped as O103:NM and the other two could not serotyped. The majority of studies performed for the detection of STEC in our country provide information about the O157 serotype; however, it is necessary to identify all strains producing stx with the multiplex PCR method as non-O157 strains may be responsible for large epidemics.

Key words: ELISA, Feces, PCR, Shiga toxin-producing *Escherichia coli*

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İnsan ve Sığır Dışkı Örneklerinde Shiga Toksin Üreten Escherichia coli Varlığının Kültür, ELİSA ve Moleküler Yöntemlerle Araştırılması

ÖZ

Shiga Toksin üreten *Escherichia coli* (STEC) 2021 EFSA verilerine göre insanlarda *Campylobacter, Salmonella* ve *Yersinia* türlerinden sonra en sıklıkla örülen dördüncü zoonotik etkendir. Hemolitik üremik sendrom (HÜS), hemorojik kolit (HK) gibi çok ciddi komplikasyonlara neden olabilmektedir. 2011 yılında Almanya'da 54 kişinin ölümüne ve birçok kişide HÜS oluşumuna sebep olan salgın gibi büyük salgınlara neden olabilir. Tanıda STEC bakterisinin tespiti ve toksin oluşumunu göstermek önemli bir yol göstericidir. Bu çalışmada, dışkı kültürü istemi yapılan hastalarda STEC sıklığının belirlenmesi ve bir entegre et tesisinde bulunan sığırlarda bu mikroorganizmanın kolonizasyon oranlarının saptanması amaçlanmıştır. İnsanlardan toplam 250 dışkı örneği ve bir entegre et tesisinden toplanan 180 sığıra ait dışkı örnekleri STEC varlığı yönünden incelenmiştir. Örnekler, CHROMagar STEC besiyeri ve MacConkey sıvı besiyerlerinde ekimi yapılmış, daha sonra enzyme linked immunosorbent assay (ELISA) ve polimeraz zincir reaksiyonu (PZR) yöntemleri kullanılarak STEC varlığı araştırılmıştır, PZR'de pozitif çıkan patojen gen bölgeleri için dizileme, kültürde üreyen STEC'ler için serotiplendirme yapılmıştır. İnsanlarda araştırılan örneklerin hiçbirinde STEC tespit edilmemiştir. Hayvan öreklerinin 11'i ELISA ile pozitif bulunmuştur. ELISA yöntemiyle pozitif bulunan örneklerin 8'i aynı zamanda PZR yöntemiyle de pozitif saptanmıştır. PZR yöntemiyle pozitif bulunan örneklerin 3'ü CROMagar STEC besiyerinde üretilebilmiştir. İzole edilen 3 bakteriden 1'i O103:NM olarak serotiplendirilmiş diğerleri serotiplendirilememiştir. Ülkemizde STEC varlığının tespiti için yapılan çalışmaların büyük bir kısmı O 157 serotipi hakkında bilgi vermektedir, ancak O 157 dışı suşlarında büyük salgınlara sebep olabilmesi nedeniyle multipleks PZR yöntemiyle, Stx üreten tüm suşların tespit edilmesi gerekmektedir.

Anahtar kelimeler: Dışkı, ELISA, PCR, Shiga Toksin üreten *Escherichia coli*

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ORCID ID; *SÜ: 0009-0002-9185-0109, HMT: 0000-0002-7651-1793*

*Corresponding author e-mail: drselahattinunlu@gmail.com

INTRODUCTION

Escherichia coli (E. coli) is generally a harmless commensal bacteria. However, some E. coli strains may cause disease if they invade intestinal mucosa, release toxins or enter blood circulation. These strains are pathogenic E. coli with the ability to cause infection (Torres2010). Shiga toxin (Stx) producing E. coli (STEC) is among these pathogens and may cause very serious complications like hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) in humans (Paton and Paton 1998). Stx is one of the most important virulence factors in pathogenesis. Stx comprises two major groups called Stx1 and Stx2 and these groups also contain several subgroups (Bergan et al. 2012). Not all Stx variants cause serious disease; only some are associated with serious disease. For example, while HC and HUS are mostly associated with Stx1a, the strains Stx1c and Stx1d are less associated with infections in humans. In the Stx2 group; Stx2a, Stx2c, Stx2d, Stx2b and Stx2e were found to be more virulent than Stx2f and Stx2g (Harada et al. 2023). STEC strains firstly colonize intestinal mucosa, then form a characteristic histopathological lesion. Formation of this lesion, called an attaching and effacing (A/E) lesion, is controlled by the locus of enterocyte effacement (LEE) pathogenicity island. The Eae gene region codes intimin and is a component of the LEE gene region (Garmendia et al. 2005; Torres2010; Prager et al. 2011). STEC secretes alpha hemolysin (hly) and this enzyme forms a cavity in the cell membrane and degrades erythrocytes. There are four genes responsible for the synthesis of alpha hemolysis; these are HlyA, HlyB, HlyC, and HlyD (WelchandPellett 1988; Welch1991; Eklund2005). For STEC infections, cattle are the most important reservoir (Torres2010). In this study, an attempt was made to detect the presence of STEC with culture, micro ELISA and multiplex classic polymerase chain reaction (PCR) methods in cattle feces and selected human feces.

MATERIALS and METHODS

Collection of Samples

A total of 250 Human fecal samples were investigated macroscopically and microscopically from July 2014 to December 2014. Samples comprised with runny/soft texture and/or containing leukocytes, erythrocytes or leukocytes and erythrocytes, watery and mucous-rich features.

Animal feces were obtained from a meat and meat products integrated facility under observation of a veterinarian. A total of 180 samples were taken, with 90 obtained in July 2014 and 90 obtained in August 2014, from the anal region of cattle using single-use clean and dry rods.

Culture

A loop of feces was taken from the collected material and firstly inoculated on CROMagar STEC medium. Then a loop of feces was inoculated to MacConkey broth were incubated at 37 °C for 16-24 hours and then examined for whether mahogany-colored colonies formed on CROMagar STEC medium. Colonies with blue color, transparent or with other colors were not accepted as STEC. *E. coli* was confirmed by definition with the Vitek 2 Compact (Biomerieux, France).

ELISA

The presence of stx1 and stx2 toxins were investigated in MacConkey broth by ELISA method, and also Samples positive for the presence of Stx with the ELISA method on MacConkey broth were investigated for colonies on CROMagar STEC medium. Bacteria producing mahogany-color colonies were tested with ELISA. The SHIGA TOXIN CHEK (Alere, USA) was used as the micro ELISA kit. The kit contain 96 wells micro ELISA plates covered with monoclonal antibodies against Stx1 and Stx2. Results were read at 450/620 nm wavelength with a spectrophotometer device (μQuant Microplate Spectrophotometer, BioTek, USA). Samples with OD >0.080 were accepted as positive.

DNA Isolation

Multiplex PCR was applied to DNA samples isolated from both MacConcey broth and mahogany colored colonies growht on the CROMagar STEC.

From MacConkey broth, 500 μl was taken and centrifuged at 10000 rpm for 5 minutes. Precipitate at the base of the tube had 500 μl sterile water added. Then it was boiled for 10 minutes at 100 °C and the tube was cooled in ice for 10 minutes. Tubes were centrifuged for 3 minutes at 14000 rpm. After the centrifuge procedure, 100 μl of the fluid remaining at the top of the tube was removed and stored in a freezer at -30 °C for use as template DNA. Nearly 60 ng DNA was detected in 1 μl of the supernatant obtained by isolation (Nano-200 Micro Spectrophotometer, China). (Hala and Ehab2010; Sánchez et al. 2010). All procedures were made for each samples.

DNA Isolation from EMB medium: The mahogany-colored colonies growht on the CROMagar STEC medium were passaged on to eosin methylene blue (EMB) medium. Based on the colony size on EMB medium, 3-5 colonies were transferred to tubes containing 100 μl sterile distilled water. The tubes were boiled for 10 minutes at 100 °C and cooled. Then tubes were centrifuged for 2 minutes at 13000 rpm and the supernatant fluid above the precipitate at the base of the tube was stored at -30 °C to be used as a template DNA. (Dastmalchi and Ayremlou 2012)

PCR

For investigate *stx1, stx2, HlyA, eae, 16 srRNA* gene regions multiplex pcr assay were used for each DNA isolates from the MacConcey broth and mahogany-colored colonies growht on the CROMagar. For the amplification procedure, a 50 µl PCR mixture was prepared. The PCR reaction was performed in a BIO-RAD ICycler Thermal Cycler. *E. coli* ATCC 43895 strain was used for positive control and *E. coli* ATCC 25922 strain was used for negative control. For the PCR mixture, 10X Taq buffer 5 µl (100 mM Tris-HCl, 500 mM KCl:Thermo Scientific, USA), 1.5 mM $MgCl_2$ (3 µl, 25 mM $MgCl_2$: Thermo Scientific, USA), 0.2 mM dNTPs (5 µl, 2mM dNTPs: Thermo Scientific, USA), forward primer 2 µl (10 pikomol.μl^{-1}: Thermo Scientific, USA), reverse primer 2 µl (10 pikomol.μl^{-1}: Thermo Scientific, USA) (Table 1), Taq DNA polymerase: 1 µl (5U: Thermo Scientific, USA), template DNA 2 µl and sterile distilled water to reach 50 µl were used.

For initial denaturation at 94 °C for 4 minutes;

40 cycles,

- 94°C (1 min denaturation),
- 48°C (1 min adhesion),
- 72°C (90 s lengthening)

After the last cycle ended, final lengthening was performed with 5 minutes incubation at 72 °C.

Table 1. Sequences used as primers and predicted length of amplification products (Schmidt et al., 1995; Wang et al., 2002; Blanco et al., 2003)

Gene region	Oligonucleotide sequence	Length of amplification product
Stx1	R:5'-CGT GGT ATA GCT ACT GTC ACC-3' F:5'-CGC TGA ATG TCA TTC GCT CTG C-3'	302 bp
Stx2	R:5'-CTG CTG TGA CAG TGA CAA AAC GC-3' F: CTT CGG TAT CCT ATT CCC GG-3'	516 bp
EHEC-HlyA	R:5'-TCT CGC CTG ATA GTG TTT GGT A-3' F:5'-GGT GCA GCA GAA AAA GTT GTA G-3	1551 bp
eae	R:5'-GCG GTA TCT TTC GCG TAA TCG CC-3' F:5'-GAG AAT GAA ATA GAA GTC GT-3'	775 bp
16S rRNA	R:5'-ACC GCT GGC AAC AAA GGA TA -3' F:5'-CCC CCT GGA CGA AGA CTG AC-3'	401 bp

R: Reverse, **F:** Forward, **bp:** base pair, ***Stx1*:** Shiga toxin 1; ***Stx1*:** Shiga toxin 2; **EHEC-*HlyA*:** Enterohemorrhagic *Escherichia coli* hemolysin A; ***eae*:** "Effacing and attaching"; **16S rRNA:** 16S Ribosomal ribonucleic acid.

Table 2. National Center for Biotechnology Information Access Numbers for positive polymer chain reaction samples

Sample Number	Positive Gene Region	NCBI Access Number
18	*eae*	KT009017
101	*Stx1*	KT009018
47	*Stx1*	KT009019
51	*Stx1*	KT009020
180	*Stx2*	KT009021
18	*Stx2*	KT009022
47	*Stx2*	KT009023
131	*Stx2*	KT009024
39	*Stx2*	KT009025
25	*Stx2*	KT009026

NCBI: National Center for Biotechnology Information, ***Stx1*:** Shiga toxin 1; ***Stx2*:** Shiga toxin 2; ***eae:*** "Effacing and attaching"

Electrophoresis and Assessment of Bands Procedure

Amplified PCR products underwent the electrophoresis procedure (Thermo scientific EC300 XL, USA) with 1.5% agarose gel (Prona, Spain) containing ethidium bromide at 100 V for 90 minutes. The electrophoresis procedure used a 100-1500 bp (GeneON, Germany) marker.

Bands forming as a result of electrophoresis were investigated with a gel imaging system (Gel Doc 2000, Bio-Rad, USA). Sequencing the amplified PCR products were sent to MedSanTek (Istanbul, Turkey). Analysis of the sequence results were performed using the DNA Chromatogram Explorer Lite V4.0.0 programe and compared with the NCBI-Nucleotide genebank database. Access numbers obtained for every gene region (Table 2).

Serotyping

The STEC colonies growht in medium and isolated were sent to the National Enteric Pathogens Reference Central Laboratory in the Turkish Public Health Institution in Ankara. Samples with agglutination to O and/or H antigens had serotypes identified and results were reported.

Statistical Analysis

Statistical analysis was performed using SPSS 21 with serial number 10240642 and Medcalc V14.12 statistical programs. Statistical analysis used the McNemar test and calculated sensitivity, specificity, positive and negative cut-off values. Descriptive statistics are given as arithmetic mean ± standard deviation. For all statistics, the limit of significance was chosen as $p<0.05$.

RESULTS

Cattle samples

Of 180 cattle feces, 11 (6.1%) were identified to be positive for Stx presence with the ELISA method. Of the 11 samples positive for Stx with the ELISA method, 8 (4.4% of the total sample number) were positive for 16S rRNA along with at least 1 positive for *Stx1, Stx2,* and *eae* gene regions with PCR (Figure 1) 6 were positive for S*tx2*, 3 for S*tx1*, and 1 was positive for *eae* with the PCR method. The number of samples positive for the *Stx2* gene region was more than for the *Stx1* region and the difference was statistically significant (p=0.0001). However, there was no statistically significant difference related to the detection rates for this pathogen between the ELISA and PCR methods (p=0.250). Colonies of three samples (1.6%) (Figure 2) were positive for the targeted pathogenicity regions with ELISA. Additionally, *E. coli* was confirmed by definition with the Vitek 2 Compact (Biomerieux, France) system and STEC was identified. Only sample no. 180 was serotyped as O103:NM, while the other 2 samples could not be serotyped (Table 3). Positive samples are summarized one-by-one with test results for each method in the table (Table 4). Of the 169 animal samples negative on the ELISA test, none of the targeted *Stx1, Stx2, eae, and EhlyA* gene regions were identified with PCR and they were accepted as negative in terms of STEC.

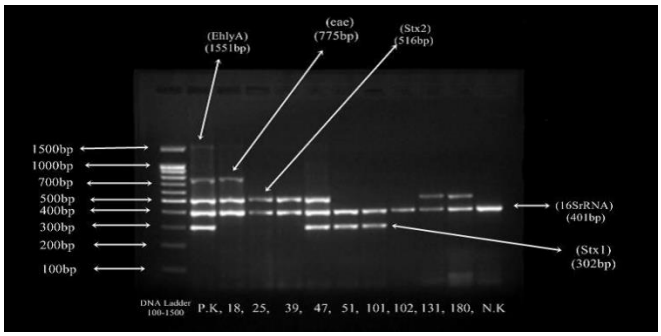

Figure 1: Appearance of agarose gel bands in sample positive according to ELISA test and polymerase chain reaction.
PK: positive control, **NK:** negative control.

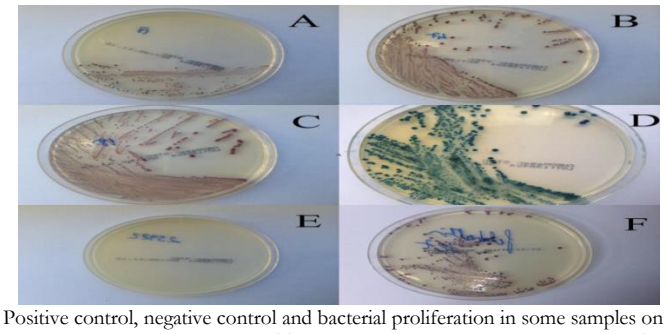

Figure 2: A-G. Positive control, negative control and bacterial proliferation in some samples on CROMagar STEC medium.
STEC positive mahogany-color colonies (A-C). STEC negative blue-color colonies (D). *E. coli* ATCC 25922 (no proliferation) (E). *E. coli* ATCC 43895 (STEC positive mahogany-color colonies) (F).

Table 3. Serotyping results for strains

No	NEPRL Protocol No	Laboratory Protocol No	Serotype
1	140583	Sample no. 18	ONT:NM
2	140584	Sample no. 47	ONT:NT
3	140585	Sample no. 180	O103:NM
4	140586	Positive control	O157:H7

NT: Non-typeable; **NM**: Non-motile **NEPRL**: National Enteric Pathogens Reference Laboratory

Table 4. Cattle feces samples with at least one positive result from the 3 methods

Sample No:	ELISA	PCR	Proliferation on CROMagarSTEC
13.	Positive	Negative	Negative
18.	Positive	Positive(16s rRNA, *Stx2, eae*)	Positive
25.	Positive	Positive (16s rRNA, *stx2*)	Negative
39.	Positive	Positive (16s rRNA, *Stx2*)	Negative
47.	Positive	Positive (16s rRNA, *stx1,Stx2*)	Positive
51.	Positive	Positive(16s rRNA, *Stx1*)	Negative
101.	Positive	Positive (16s rRNA, *Stx1*)	Negative
102.	Positive	Negative(only 16s rRNA positive)	Negative
129.	Positive	Negative	Negative
131.	Positive	Positive (16s rRNA, *Stx2*)	Negative
180.	Positive	Positive (16s rRNA, *Stx2*)	Positive

Stx1: Shiga toxin 1; ***Stx2***: Shiga toxin 2; ***eae***:"Effacing and attaching"; **16S rRNA:**16S ribosomal nucleic acid

Human samples

There were 143 men and 107 women among the 250 patients with fecal samples investigated. The general mean age was 23.2 years, with the mean age for women 25.2 years and mean age for men 21.2 years. Of the samples, 108 were obtained from the pediatric emergency clinic, 86 from the adult emergency clinic, and the remaining 56 samples from a variety of wards and clinics.

Direct microscopic examination of fecal samples found abundant leukocytes in 98 (39.2%), rare leukocytes in 42 (16.8%) and very rare leukocytes in 26 (10.4%). Of the total of 166 samples (66.4%) with leukocytes identified, 57 also had erythrocytes observed (22.8%). Macroscopic investigation found 161 of the samples (64.4%) had mucous and/or liquid appearance, while the remaining 89 samples (35.6%) had soft texture. Tests found negative for the presence of STEC by PCR, ELISA and culture methods. Of the samples, 25 were identified to have other enteric bacteria including 14 *Salmonella* spp., 10 *Campylobacter* spp. and 1 *Shigella* spp.

DISCUSSION

Cattle appear to act as a significant reservoir for STEC infections occurring in humans. STEC colonization of these animals may reach 60%, while studies generally found the average rate was 10-25%. Carriers of O157 serotype are very rare compared to these rates; for example, this serotype was found in 0-2.8% of milk and meat cattle in the United States of America (USA) (Harada et al. 2023).In studies about animals in Türkiye, a study encompassing investigation of 1000 water buffalo feces in Samsun isolated 38 *E. coli* O157:H7 strains (3.8%) (Nuhay and Gülhan 2017). A study by Ayaz et al. in 2014 in the Kırıkkale region obtained carcass swabs, rectoanal mucosal swabs and bile samples from 240 cattle and isolated *E. coli* O157:H7 in 6.3% of swab samples (Erol2016). Another study performed in the Afyonkarahisar region in February-August 2014 on 237 cattle feces samples found this rate was 4.6% by PCR method (Aslan et al. 2016). A study in Bursa in 2014 found STEC in 6.3% of cattle (Ahmed 2017). Contrary to the low positivity rates in other studies, it was reported that *E. coli* O157 was isolated in 13.6% (77 samples) of rectal swab samples taken from 565 cattle carcasses mainly from Hatay but also Adana, Kahramanmaraş, and Mersin (Aslantaş et al. 2006).

There is little research about identifying the STEC colonization rates in cattle in our country. When the available studies are examined, some had no positivity

identified while some detected high rates like 13.6% positivity. In our study, a total of 8 animals were found to be positive STEC carriers with both ELISA and PCR methods, though none of the 3 isolated STEC strains were found to be O157:H7 according to serotyping results. The difference in findings obtained as a result of studies is considered to be due to geographical differences, isolation and definition methods not being the same and seasonal differences.
The number of studies performed to determine the prevalence of STEC strains apart from O157 is at lower levels compared to O157, which is linked to the later understanding of the importance of these strains. The prevalence of non-O157 strains is generally ignored; however, non-O157 serotypes were isolated in 25% of people developing HUS. (WHO 1998). According to the European Food Safety Authority (EFSA) data for 2012, 3316 cattle were investigated for the presence of STEC and 195 were identified to be positive (5.9%). Of the 129 cattle samples that could be serotyped, 13 different O serotypes were found (EFSA 2022). In Türkiye, the studies to determine the prevalence of STEC in cattle were performed to identify the O157:H7 strains, as in many other countries. For this reason, the non-O157 STEC prevalence rates are not known. In this study, of the 3 strains isolated from 180 samples, only 1 (0.5%) was serotyped as O103:NM (non-motile). A study in 2013 from Greece investigated 140 cattle with both ELISA and PCR methods. They found 4 samples positive according to ELISA (2.9%) and 2 samples positive with PCR (1.4%). The 2 samples positive with PCR were serotyped as O157:H7. In the study, they emphasized that the PCR method was a more reliable method to identify STEC (Pinaka et al. 2013).
In our study, 8 out of 11 samples positive according to ELISA were identified to have *Stx1* and/or *Stx2* gene regions with PCR, while the other 3 samples were negative. It is considered that the ELISA method provided false positive results for the 3 negative samples. Different studies have stated that ELISA tests may provide false positive results (Ball et al. 1996; Pulz et al.2003). The sensitivity, specificity, positive and negative predictive values for the ELISA test compared to PCR were 100%, 98.26%, 72.73% and 100%, respectively. In our study, among 180 animal feces, the 8 samples consistent with STEC that were positive with PCR and also had sequencing results compared to the database is equivalent to 4.4%. The studies performed in Türkiye to determine STEC prevalence mainly used SMAC medium and defined the strains according to whether O157 agglutinated with antiserum by choosing sorbitol-negative colonies. For this reason, most research only provides information about the frequency of O157. There is not much data about both the O157 and non-O157 STEC colonization rates. In this study, instead of SMAC agar or CT-SMAC agar, STEC strains apart from O157 were produced using the CROMagar STEC medium, with differentiation ability. However, only 3 of the 8 samples positive for STEC with PCR (38%) could be isolated from this medium. This difference is thought to be due to the low STEC bacteria counts in the inoculated samples and lack of growth of this bacteria in the medium, even though PCR may identify positive samples by providing sensitive results in the presence of very little bacteria.
The incidence of STEC in humans is different from country to country. According to CDC FoodNet data, the STEC incidence in the USA was reported as 1.5, 1.69 and 1.81 for the years 2015, 2016 and 2017 representing 4824, 5443 and 6034 cases, respectively (CDC 2021). According to EFSA data, the STEC incidence was 1.9, 1.5 and 2.1 for 2019, 2020 and 2021, respectively. For the same years, 7801, 4489 and 6084 cases were reported (EFSA2022). Studies of humans in our country, similar to animal studies, dominantly used SMAC medium and chose sorbitol-negative colonies with detection according to agglutination with O157 antiserum. For this reason, most research only provides information about the O157 strains. The results of different studies in Türkiye found the STEC O157:H7 incidence was between 0 and 4% (Ünlü 2015).
It is known that non-O157 serotypes were identified in some studies in recent years. In 2011 an epidemic due to O104:H4 serotype occurred in Europe, led by Germany, and caused 3816 cases with 845 of these cases (22%) developing HUS and the death of 54 patients reported (Frank et al. 2011). The effect of this epidemic in Türkiye was an increase in HUS in pediatric patients in the same year. Of 70 patients with HUS diagnosis treated in a total of 40 pediatric centers, only 4 were serotyped and 2 were O104:H4 (Ekinci at al. 2013). A study of fecal samples obtained from children with suspected HUS from 2012 to 2019 identified STEC in 46 patients. Of these 15 were O145 serotype (32.6%) and 8 were O157 serotype (17.3%) (Okumuş 2021). A study investigating 395 samples sent to the Public Health Reference Laboratory for suspected HUS from 2011 to 2014 identified STEC in 28 samples. Among these samples, the dominant serotypes were O104 for 7 samples and O26 for 6 samples (Gulesen et al.2016). In our study, none of the 250 fecal samples from humans had O157:H7 or non-O157 STEC strains identified. The reason for this may be the low prevalence of bacteria in the region, probable antibiotic use and patients attending hospital late after diarrhea begins. In fact, it is reported that the rate of identification of this bacteria reduces as time passes after diarrhea begins (Rosensweig andGourley 1991).

CONCLUSION

For the detection of STEC, due to the difficulty in isolating this bacteria with culture methods and the high cost of ELISA tests and the antiserums used in the agglutination method, the use of molecular methods in future research will be both rapid and more

beneficial. With this aim, the use of the multiplex PCR method can be said to be appropriate in terms of both sensitivity and specificity. Additionally, serotyping is necessary for bacteria in positive samples for gene regions causing virulence, especially in epidemiological studies. As this study is the first and only study in the region, there is a need to perform more studies on both human and animal samples to determine prevalence.

Conflict of interest: The authors have no conflicts of interest to report.

Authors' Contributions: SÜ and HT contributed to the project idea, design and execution of the study. SÜ contributed to the acquisition of data. SÜ analysed the data. SÜ drafted and wrote the manuscript. SÜ and HT reviewed the manuscript critically. All authors have read and approved the finalized manuscript.

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