



DOI: 10.1515/rrlm-2017-0025

The frequency of shiga-like toxin (*stx1* and *stx2*) and EHEC-*hlyA* in food by multiplex PCR

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Abstract

Aim: The aim of the present study was to determine the frequency of shiga-like toxin (*stx1* and *stx2*) and drug resistance profiles food-borne *Escherichia coli* O157:H7 in Hatay province, Turkey.

Methods: The presence of the virulence genes (*stx1*, *stx2*, *hlyA*) in a total of 150 *E. coli* isolates were studied with multiplex PCR.

Results: A total of 327 salad samples were analyzed. *E. coli* O157:H7 was detected in 150 (45.8 %) out of 327 analyzed samples. Of these 150 isolates, the presence of *hlyA* gene was detected in 32 (21.3%) *E. coli* isolates. A total of five (15.6%) isolates in this 32 *hlyA* positive isolates had *stx2* gene, two (6.3%) of them had *stx1* gene and one (3.1%) of the isolates was found to be positive for both *stx1* and *stx2* genes. It was found that all *E. coli* O157:H7 isolates were resistant to erythromycin. While the highest rate of antibiotic resistance was observed for ampicillin (68.8%), no antibiotic resistance against cefuroxime, ciprofloxacin and cephalosporins was identified.

Conclusions: The results obtained in our province showed that *E. coli* strains isolated from salad samples were found to have some important virulence genes such as *stx1*, *stx2*, and *hlyA*. The *stx2* frequency was found to be higher than *stx1* frequency. Also, it was observed that there was not any significant correlation between drug resistance profiles and presence of toxin genes in *E. coli* O157:H7 strains. As a result, increasing frequency of STEC O157 serotype among foodborne pathogens is a growing public health problem.

Keywords: *E. coli* O157:H7, *stx1*, *stx2*, *hlyA*, PCR

Received: 2nd March 2017; Accepted: 2nd July 2017; Published: 17th July 2017

Introduction

A significant part of salad microflora is composed of members of Enterobacteriaceae family. It is reported that the number of coliform bacteria in salads may reach 10⁵/g. Particularly, *E. coli* is one of the most isolated bacteria in salads (1-4).

E. coli shows many different serotypes and of these serotypes, *E. coli* O157:H7 is a significant morbidity and mortality cause for human health. Bacteria enterohemolysin (*hlyA*) and cytotoxin known as shiga toxin are important virulence factors (5). Enterohemolysin is coded by *hlyA* and causes lysis of erythrocytes, contributes to

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virulence of microorganisms and thus provides iron sources to bacteria so that they can survive in the intestine. As for shiga toxins, they have the ability to inhibit host protein synthesis and may lead to eucaryotic cell injury and death (6).

Infections caused by *E.coli* O157:H7 serotype may range from mild infections such as watery diarrhea to diseases such as hemorrhagic colitis, hemolytic uremic syndrome (HUS) and thrombocytopenia. HUS is considered as the primary reason for acute renal insufficiency among children. The treatment of the disease includes hemodialysis and supportive care only (7,8).

It is reported that *E.coli* O157:H7 commonly colonize in the intestinal system of farm animals. In recent epidemiological studies, it is indicated that dairy cattle are primary carriers of *E.coli* O157:H7 (9-11).

It is indicated that most of the *E.coli* O157:H7 infections may be associated with poorly cooked meat and dairy products, not-pasteurized products and vegetables contaminated with feces of these farm animals. Furthermore, it is pointed out that contamination risk by humans may be possible, too (12).

Because microorganisms exist in low quantities in contaminated food samples or feces samples, there may be problems and limitations in diagnosis using conventional methods. Although different methods such as culture tests and enzyme-linked immunosorbent assays (ELISA) have been used to detect bacteria, today molecular techniques based on polymerase chain reaction (PCR) are preferred for accurate and fast diagnosis of *E.coli* O157:H7 due to the problems related with practicabilities and sensitivities of these above mentioned methods (13,14).

Following isolation of microorganisms, it is very important to demonstrate presence of virulence genes which are indicators of pathogenicity. Today; multiplex PCR are chosen most frequently for the diagnosis of important

virulence genes such as *E.coli* O157:H7 shiga toxin-1 (*Stx1*), shiga toxin-2 (*Stx2*) and *hlyA* (11,15). Food borne *E.coli* O157:H7 prevalence ranges from 0.8% to 22.5% in various studies conducted all over the World (14,15). In Turkey, there are studies that report that food borne *E.coli* O157:H7 prevalence ranges between 1% and 7.6% (15,16).

The aim of this study was to identify prevalence of *E.coli* O157:H7 in salad samples and presence of enterohemolysin *hlyA*, *Stx-1* and *Stx-2* toxins in *E.coli* O157:H7 strains using multiplex PCR and to determine whether or not restaurants serving in Hatay Province (South of Turkey) are a potential threat to public health.

Materials and Methods

In the study, 327 salad samples taken from various restaurants and snack bars located in South of Turkey (Hatay Province) were included. According to Food Codex and Codex Alimentarius, 150 of 327 *E.coli* isolates were found to exceed the limit of pathogenicity. These strains were examined (17).

These isolates were evaluated in terms of *E.coli* O157:H7 and presence of *hly-A*, *stx1* and *stx2* virulence factors. Salad samples of 250 gr. were collected in sterile containers by paying attention to aseptic conditions right after they had been prepared for service. Salad samples were thoroughly mixed before they were aseptically collected. Then, they were delivered to bacteriology laboratory through a cold chain. The samples were immediately homogenized and samples weighed as 1 gr. were added to the Mueller-Hinton Broth (Merck, Germany) for proliferation and incubated for 48 hours at 37°C. Following the 48 hours of incubation; samples of 100 µl were taken from the broth media using micropipettes and transferred into MacConkey or EMB (eosin methylene blue) agar. Plates were incubated for 48 hours at 37°C and analyzed.

Isolation and Identification

Some inclusion and exclusion criteria have been taken into consideration in the identification of *E. coli* isolates. Inclusion criteria for *E. coli* isolates were growth on MacConkey or EMB agar medium (HiMedia, India) and complying with standard biochemical tests including triple sugar iron agar (TSI), sulfide indole motility (SIM), citrate agar, and methyl red voges-proskauer (MRVP), (18). The exclusion criteria were inhibition of growth on MacConkey or EMB agar medium and inconsistencies in the results of biochemical tests for *E. coli*.

Identification of the isolates was confirmed with Vitek-2 system (bioMérieux, France). *E. coli* isolates were kept at -70 °C in *broth containing 20% glycerol*. Then, the collected samples in deep freeze were inoculated on *sorbitol-MacConkey* (SMAC) agar. After at least 24 hours of incubation at 37°C, sorbitol-negative colonies were identified as O157:H7 isolates. All sorbitol-negative isolates were confirmed by O157 antisera (Difco Laboratories).

DNA Extraction and Multiplex PCR

For the genomic DNA extraction, commercially available GF-1 Bacterial DNA Extraction Kit (Vivantis, US) was used.

Using *hlyA*, *stx-1*, *stx-2* primers extracted from DNA samples; PCR amplifications were performed in a thermal cycler (Table I). The PCR

amplification was carried out in a total volume of 25 µl. The PCR mixture was constructed as follows: 2.5 µl PCR buffer (1x without MgCl₂, 4 µl MgCl₂ (25 mM), 1 µl dNTP (200 µM each nucleotides), each primers (both *stx-1* and *stx-2* and *hlyA* genes), Taq DNA polymerase 0.5 µl U (5 U/µl), template DNA 1 µl (approximately 50 ng). The *hlyA* gene was amplified in a single PCR reaction in one tube. The PCR mixture was exactly the same as indicated above for *stx-1* and *stx-2* genes.

In the current study; *Escherichia coli* O157:H7 ATCC 43888 was used as negative standard strain while ATCC 43895 (positive for *stx-1* and *stx-2* genes) and ATCC 43889 (negative for *stx-1*, positive for *stx-2*) were used as positive standard strain.

Standard strains were obtained from Istanbul University, Department of Pharmaceutical Microbiology of The Faculty of Pharmacy and Cukurova University, Central Laboratory Culture Collection of Medicine Faculty.

The PCR processes in Table II were applied for the amplifications of toxin genes and the presence of amplifications were examined by running them in 2% of agarose gels (Table II).

Antimicrobial Sensitivity Tests

Antimicrobial resistance profiles of sorbitol-negative isolates were studied. The susceptibility test was performed according to the Clinical and Laboratory Standards Institute (CLSI)

Table I: Oligonucleotide primers used in the study

Primer	Target gene	Primer sequence (5'-3')	Fragment size (bp)	Reference
HlyA	hlyA-F	GTA GGG AAG CGA ACA GAG	361	Wang et. al., 1997
HlyA	hlyA-R	AAG CTC CGT GTG CCT GAA	361	Wang et. al., 1997
Stx1	stx1-F	ACA CTG GAT GAT CTC AGT GG	614	Manna et. al., 2006
Stx1	stx1-R	CTG AAT CCC CCT CCA TTA TG	614	Manna et. al., 2006
Stx2	stx2-F	CCA TGA CAA CGG ACA GCA GTT	779	Manna et. al., 2006
Stx2	stx2-R	CCT GTC AAC TGA GCA CTT TG	779	Manna et. al., 2006

Table II. Thermal cycles for three primers used in the study

No	Step	hly-A (<i>E.coli</i> O157:H7)	stx-1 and stx-2 (STEC)
1	Initial denaturation	94°C / 4 min.	94°C / 4 min.
2	Denaturation	94°C/ 45 sec.	94°C/ 45 sec.
	Annealing	61°C/ 1 min. 30 cycle	61°C/ 1 min. 30 cycle
	Extension	72°C/ 1 min.	72°C/ 1 min.
3	Final extension	72°C/ 7 min.	72°C/ 7 min.
4	Keeping	+4°C/ ∞	+4°C/ ∞

guidelines. Antimicrobial resistance profiles of sorbitol-negative isolates were studied. The following antibiotics were used: ampicilin (10 µg), gentamicin (10 µg), cefuroxime (30 µg), tetracycline (30 µg) and ciprofloxacin (5 µg), nalidixic acid (30 µg), cotrimoxazole (25 µg), and chloramphenicol (30 µg) (Oxoid, England). The results were interpreted according to CLSI (2015) criteria (19).

Statistical analysis: All data were evaluated by χ^2 test using Statistical Package for Social Sciences (SPSS1 for Windows V. 17.5, Chicago, USA) software.

Results

In 150 of the 327 salad samples, significantly *E.coli* reproduction was determined more than the reference value set by Food Codex, Microbiological criteria. A total of 150 *E.coli* isolates were tested with sorbitol MacConkey agar and

32 (21.3%) isolates were sorbitol-negative. Serologically, all of the 32 sorbitol negative isolates were confirmed as serotype O157:H7. Presence of *E.coli* O157:H7 was confirmed with presence of *hly-A* gene of the microorganism. It was found out among the isolates identified as *E.coli* O157:H7 that 15.6% (5/32) had *stx2* toxin genes, 6.3% (2/32) had *stx1* toxin genes and 3.1% (1/32) had both *stx1* and *stx2* toxin genes (Figure 1 and 2).

In our study; 21.3% (32/150) of 150 *E.coli* isolates isolated from salad samples showed the presence of *E.coli* O157:H7 with *hlyA* gene positive and rate of shiga toxin *stx2* (3.3%; 5/150) was significantly higher than shiga toxin *stx1* (1.3%; 2/150), ($p < 0.05$). Also; there was an isolate that carried both *stx1* and *stx2* toxin genes (0.7%; 1/150) despite being at low level. When the rate of toxin gene carriage of *E.coli* O157:H7 isolates was assessed, it was detected that 15.6% of the isolates carried *stx2*, 6.3% of isolates carried *stx1*

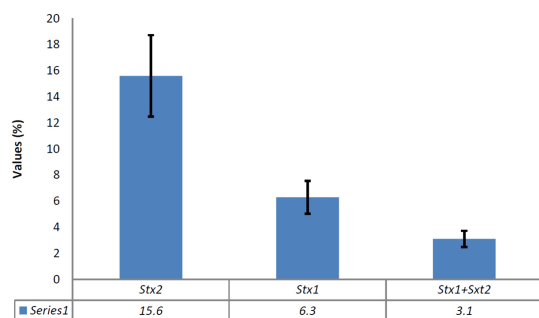


Figure 1. Incidence of shiga toxin genes (*stx1* and *stx2*) in *E.coli* O157:H7 isolates.

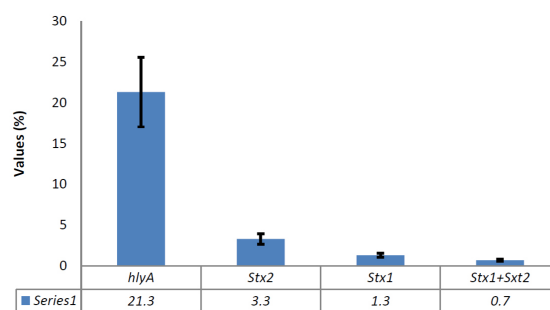


Figure 2. Incidence of *hlyA*, *stx1* and *stx2* toxin genes in *E.coli* isolates.

toxin genes and rate of isolates being positive in both *stx1* and *stx2* toxin genes, was 3.1%.

Following PCR amplifications of *hly-A*, *stx1* and *stx2* genes in *E.coli* O157:H7 isolates, the PCR products were imaged by running them in 2% of agarose gels. Electrophoresis images of these genes (*hly-A*, *stx1* and *stx2*) are presented below (Figure 3 and 4).

Antibiotic sensitivity rates and antibiotic sensitivity distribution of 32 *E.coli* O157:H7

isolates that were *hlyA* positive were demonstrated in Table 3.

It was noted that the highest resistance was against ampicillin (68.8%; 22/32) while no antimicrobial resistance occurred against cefuroxime and ciprofloxacin. It was detected that all the strains were sensitive against these two antibiotics (cefuroxime and ciprofloxacin). Furthermore, antimicrobial resistance rates against gentamicin and chloramphenicol were determined as 6.3%

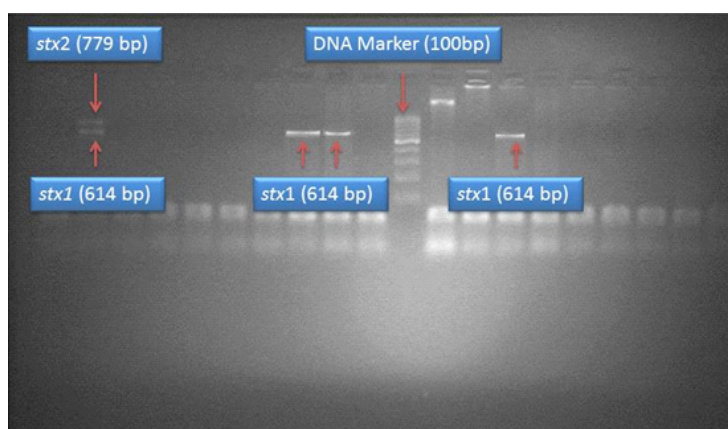


Figure 3. Agarose gel electrophoresis images of PCR amplification products of *stx1* (614 bp) and *stx2* (779 bp) genes. DNA marker (100 bp ladder) *stx1* (614 bp), *stx2* (779 bp).



Figure 4. Agarose gel electrophoresis images of PCR amplification products of Hly-A (hemolysin A) gene M: 100 bp DNA Ladder. HlyA (361 bp): 14-18 bands. Negative control: 19. and 20. bands. Positive control: 13. band.

Table III. Antibiotic sensitivity percentages of *E.coli* O157:H7 strains that were hlyA positive (n=32).

Antibiotics	Sensi- tive	Interme- diate	Resis- tant
Ampiciline	12.5	18.8	68.8
Nalidixic Acid	90.6	0	3.1
Chloramphenicol	90.6	3.1	6.3
Cotrimoxazole	93.7	0	3.1
Gentamicin	93.7	0	6.3
Tetracycline	96.9	0	3.1
Cefuroxime	100	0	0
Ciprofloxacin	100	0	0

(2/32), while the resistance rates for tetracycline, nalidixic acid and cotrimoxazole were found to be 3.1% (1/32), (Figure 5).

In Table IV, antimicrobial drug resistance patterns against ampicillin, gentamicin, cefuroxime, tetracycline, ciprofloxacin, nalidixic acid, cotrimoxazole and chloramphenicol were shown in terms of *stx1*, *stx2* and both *stx1* and *stx2* genes of positive strains of *E.coli* O157:H7 isolates.

Resistance against ampicillin, gentamicin and chloramphenicol was detected in two of the

E.coli O157:H7 strains (6.3%) that were positive in *stx1* gene, while these strains were found to be sensitive to cefuroxime, tetracycline, ciprofloxacin and nalidixic acid and cotrimoxazole. Among five *E.coli* O157:H7 (15.6%) that were positive in *stx2* gene, one strain of five isolates (20%; 1/5) have ampicillin sensitivity but the other four strains (80%; 4/5) were found to be ampicillin resistant. However, it was detected that strains that were positive in *stx2* genes were sensitive to all the other antibiotics (gentamicin, cefuroxime, tetracycline, ciprofloxacin, nalidixic acid, cotrimoxazole and chloramphenicol).

It was observed that the antimicrobial resistance profile of one strain (3.1%; 1/32) that was positive both in *stx1* and *stx2* genes (%3.1; 1/32) was completely the same as the antimicrobial drug resistance profile of the strain that was positive in *stx1* toxin only. It was noted that this strain was resistant to ampicillin, gentamicin and chloramphenicol while it was sensitive to all the other tested antibiotics.

Discussion

Diseases caused by STEC strains are an important public health problem. Although other

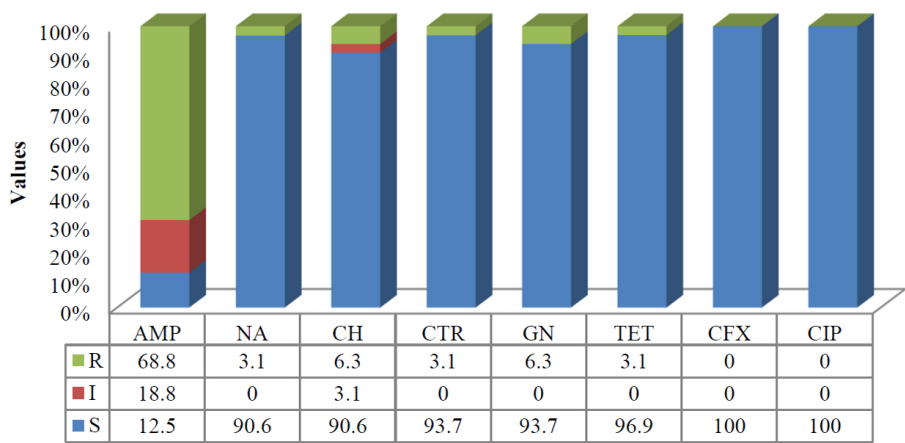


Figure 5. Antibiotic Resistance Patterns of *E.coli* O157 H7 isolates.
Abbreviations: AMP: Ampicillin; CFX: Cefuroxime; GN: Gentamicin; TE: Tetracyclin;
CIP: Ciprofloxacin; NA: Nalidixic acid; CTR: Cotrimoxazole; CH: Chloramphenicol.

Table IV. Antimicrobial drug resistance patterns in positive *E.coli* O157:H7 strains in terms of Shiga toxins.

Sample number	CTR	AM	GN	CFM	TE	CIP	CH	NA
Stx1 positive								
1	S	R	R	S	S	S	R	S
2	S	R	R	S	S	S	R	S
Stx2 positive								
1	S	R	S	S	S	S	S	S
2	S	R	S	S	S	S	S	S
3	S	S	S	S	S	S	S	S
4	S	R	S	S	S	S	S	S
5	S	R	S	S	S	S	S	S
Stx1 and Stx2 positive								
1	S	R	R	S	S	S	R	S
Total resistance percentages	100%	87.5%	0	0	0	0	0	37.5%

Abbreviations: AMP: Ampicillin; CFX: Cefuroxime; GN: Gentamicin; TE: Tetracyclin; CIP: Ciprofloxacin; NA: Nalidixic acid; CTR: Cotrimoxazole; CH: Chloramphenicol.

strains of EHEC are frequently reported to be isolated generally in water borne epidemics, it is stated that STEC is the main reason of epidemics in America, Europe and Japan. It is reported that most of the strains of these microorganisms secrete *stx2*, some of them secrete *stx1* and 2 and a few secrete only *stx1* (20-22). Most of the strains of these microorganisms secrete *stx2*, some of them have both *stx1* and *stx2*, and some of them have only *stx1* secretion.

Stx1 and *stx2* are the two most important virulence factors for humans. Studies have reported that *stx2* is a more important virulence factor than *stx1*, which is associated with human diseases. It has been reported that *stx2* is more virulent than *stx1* in animal studies (23).

In our study, the *stx2* gene frequency was found to be 15.6% (5/32) while the *stx1* toxin gene frequency was 6.3% (2/32) in sorbitol negative *E.coli* strains. Our findings displayed that 5 of 7 (71.4%) STEC O157 isolates contained the *stx2* gene. Despite that, only 2 (28.6%) of these isolates contained the *stx1* gene. Our results seem to be concordant with the literature.

Similar results have been reported from Iran by Akhi et al (90.9%), America by Mellor et al. (73%) and Argentina by Leotta et al. (91%). These findings indicate that *Stx2* is the most important virulence factor in foodborne STEC O157 infections in Turkey. Therefore, rapid and accurate detection of this toxin (*stx2*) in food is very important in preventing STEC infections (24-27).

Incidence of *E.coli* O157:H7 were investigated in various studies in Turkey and a study done in 2013 reported that *Escherichia coli* O157:H7 was detected in 5 (1%) samples including two diced meat, one minced meat and two raw-milk cheese in 500 samples taken from diced meat, minced meat, burger, raw cow's milk and raw cow's milk cheese. It was seen that 3 of these isolates (60%; 3/5) were positive in *stx1*, *stx2* and *hlyA* genes. Interestingly the study indicated that *stx1*, *eaeA* and *hlyA* genes isolated from 2 strains were obtained from raw milk products (16).

In another study done in 2012, *E.coli* incidence was found to be 36.7% in such com-

mercial cheeses consumed in Istanbul as white cheese, tulum cheese, hellim cheese but none demonstrated *E.coli* O157:H7 (21). Considering the incidence of EHEC in our study to be 21.3%, it is understandable that commercial salads to be consumed are high-risk food for EHEC infections. The study of Bingol et al. indicates that boiled dairy products are among safe products in terms of EHEC (21).

In another study about the frequency of *E.coli* O157:H7 in meat products in Eastern Anatolia Turkiye, 120 samples of bovine meat, 105 samples of chicken and turkey meat were analyzed and presence of *E.coli* O157:H7 in bovine meat, chicken and turkey meat were 3.3%, 1.6% and 2.7%; respectively (15).

Although it is reported that various contaminated animal products such as bovine meat, milk and dairy products are crucial in disease transmission, it is reported that most of the foodborne epidemic are caused by consumption of lettuce, spinach and other vegetables. According to CDC reports, 41% of 183 *E.coli* O157:H7 epidemic occurring from 1982 to 2002 was caused by steak and 21% was due to different animal products (26-29).

Consumption of green leafy vegetables has been shown to be one of the most important causes of EHEC (*E. coli* O157:H7) infections (30,31). In a study done by Wang et al. 2001, it was identified that *E. coli* O157:H7 is able to survive in rotten parts of green leafy vegetables stored at different temperatures. It was identified that pathogens injected into rotten parts of coriander survived more than four days at 8-15 °C and reproduced at 12 °C. In another study conducted by Allende et al. in 2006; viability of *E.coli* O157:H7 injected into 6 different fresh plants was analyzed and it was demonstrated that *E.coli* O157:H7 could survive at +4 °C for up to 19 days. Besides, Chauret's study in 2011 detected that 6 EHEC strains with defective *rpoS* genes became resistant against acidic environ-

ment on lettuce leaves when they were stored at ≥ 15 °C (32,33).

One of the most commonly observed characteristics of *E.coli* O157:H7 is that it is resistant against low pH broth and acidic environments that contained < 2 pH values with synthetic gastric juice. This characteristic of *E.coli* O157:H7 results in disease risk among those consuming low pH products. As emphasized in many studies, this characteristic of *E.coli* O157:H7 is used to differentiate it from other bacteria living in the microflora (29,33,34).

It was observed that there was not any significant correlation between drug resistance profiles and presence of toxin genes in *E.coli* O157:H7 strains ($p > 0.05$). Therefore, more comprehensive studies are required to reach a conclusion on this subject.

Conclusion

In this study, the *stx2* frequency was found to be higher than the *stx1* frequency. As a result, increased frequency of STEC O157 serotype among foodborne pathogens is a growing public health problem. In light of the study results, it was established that salad samples contained pathogenic *E.coli* agents that may be dangerous for human health. Because *E.coli* O157: H7 can lead to serious infections for human health such as hemorrhagic colitis or hemolytic uremic syndrome, we think that cleaning procedures, good manufacturing and storing practices should be established to prevent O157H7 infection.

Besides this, although resistance to ampicillin in *E. coli* O157:H7 isolates was found to reach important proportions in the study, resistance rates to other antibiotics were not very high. In developing countries such as Turkey, the fact that *E. coli* O157:H7 reports are very limited indicates the necessity of examining this pathogen in more detail. Thus, it is thought that significant epidemiological data can be obtained for our country.

Acknowledgment

This work was supported by the Research Fund of Mustafa Kemal University. This study has been in accordance with both legal and ethical grounds.

Conflict of interest

No conflict of interest to declare.

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