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**RESEARCH ARTICLE**

# Seasonal Distribution and Virulence Properties of *Escherichia coli* O157, *Escherichia coli* O157:H7 Isolated from Minced Meat and Traditional Cheese Samples[#]

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**ABSTRACT**

This study was carried out to investigate the presence and virulence properties of *Escherichia coli* O157, *Escherichia coli* O157:H7 in some foods of animal origin (minced meat, Carra and Surk cheeses) and to determine the monthly and seasonal distribution of the isolates. A total of 71 traditional cheeses (35 Surk, 36 Carra) and 60 minced meat samples were collected. Immunomagnetic separation based cultural technique and PCR were used for the isolation and identification of *E. coli* O157:H7. Overall, 17 (13%) and 16 (12.2%) of 131 food samples were found to be contaminated with *E. coli* O157 and *E. coli* O157:H7, respectively. Intimin was determined as the most common virulence factor, since the majority (83.3%) of isolates harbouring the *eaeA* gene. $Stx_2$ gene was only detected in two (6.6%) isolates recovered from minced meat samples. In this study, isolates were obtained from the samples at most in spring. These results indicate that the presence of virulent *E. coli* O157:H7 strains in minced meat and traditional cheeses can be a potential risk for human infections.

**Keywords:** *E. coli* O157:H7, Immunomagnetic separation, PCR, Virulence.

**Kıyma ve Geleneksel Peynir Örneklerinden İzole Edilen *Escherichia coli* O157, *Escherichia coli* O157:H7'nin Virülens Özellikleri ve Mevsimsel Dağılımı**

**ÖZ**

Bu çalışma, bazı hayvansal gıdalarda (kıyma, Carra ve Surk peynirleri) *Escherichia coli* O157, *Escherichia coli* O157:H7varlığı ile virülens özelliklerini araştırmak ve izolatların aylık-mevsimsel dağılımlarını belirlemek amacıyla yürütüldü. Toplam 71 geleneksel peynir (35 Surk, 36 Carra) ve 60 kıyma örneği alındı. *E. coli* O157:H7'nin izolasyon ve identifikasyonunda immunomanyetik separasyon bazlı kültür tekniği ve PCR kullanıldı. Genel olarak, 131 gıda örneğinin 17 (% 13) ve 16'sının (% 12.2) sırasıyla *E. coli* O157 ve *E. coli* O157:H7 ile kontamine olduğu tespit edildi. İzolatların çoğunluğunun (% 83.3) *eaeA* genine sahip olduğu ve intimin en yaygın virülens faktörü olarak belirlendi. $Stx_2$ geni yalnızca kıyma örneklerinden elde edilen iki (% 6.6) izolatta saptandı. Bu çalışmada, izolatlar örneklerden en fazla ilkbaharda elde edildi. Bu sonuçlar kıyma ve geleneksel peynirlerde virülens özelliği olan *E. coli* O157:H7 suşlarının bulunmasının insanlardaki infeksiyonlar açısından potansiyel bir risk oluşturabileceğini göstermektedir.

**Anahtar Kelimeler:** *E. coli* O157:H7, İmmunomanyetik separasyon, PCR, Virülens.

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## INTRODUCTION

*Escherichia coli* is found in the normal intestinal flora of humans and warmblooded animals. The bacterium can be spread out through feces and contaminate food in various ways and cause foodborne infections. Therefore, the presence of this bacterium in food is considered to be an indicator of fecal contamination (Clermont et al. 2000). Pathogenic *E. coli* strains are classified into six pathotypes based on their virulence characteristics, pathogenicity mechanisms, clinical syndromes, and differences in O:H serotypes. These are enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), diffuse-adhesive *E. coli* (DAEC), enteroaggregative *E. coli* (EAEC) and enterohemorrhagic *E. coli* (EHEC) (Meng et al. 2007).

The most important serotype among the enterohemorrhagic *E. coli* (EHEC) strains is *E. coli* O157: H7, causing foodborne infections which can result in death. *E. coli* O157:H7 was first introduced to be a foodborne pathogen in 1982 as the cause of two outbreaks in the United States. Contaminated hamburger served as undercooked has been found to be responsible for these outbreaks. Today, *E. coli* O157:H7 has been reported to cause serious health problems worldwide, particularly in Europe, America, South Africa and Japan (Maurer et al. 1999, Fratamicoet al. 2000, Hodges and Kimball 2005).

The virulence of *E. coli* O157: H7 is very high, and a few of these bacteria (10-100) ingested with food can cause the symptoms to occur in affected individuals. Also, *E. coli* O157:H7 is resistant to acidic conditions and can retain its vitality for a long time during frozen storage (Menget al. 2007).

The most important source of *E. coli* O157:H7 infections in humans is infected cattle. Epidemiological studies have revealed that the infection is generally caused by the consumption products provided from cattle, such as undercooked minced meat and raw milk and dairy products. In addition, the incidence of foodborne infections caused by *E. coli* O157:H7 has been reported to reach a higher level during the warmest months of the year (Paton and Paton1998, Rey et al. 2006, Meng et al. 2007, Perelle et al. 2007).

Genetically, *E. coli* serotypes have pathogenicity island known as the locus of enterocyte effacement (LEE). Stxs (shiga-like-toxin; Stx1 and Stx2) are the most important virulence factors in pathogenicity of *E. coli* O157:H7. Stx1 and Stx2 toxins are encoded by $stx_1$ and $stx_2$ genes, respectively. The main receptors for these toxins are globotriaosylceramide ($Gb_3$) found in kidney epithelial cells. Also, intimin and enterohemolysin are among the virulence factors that are important for bacteria. Intimin is an outer membrane protein that allows the bacterium to adhere and attach to the host mucosal surfaces and encoded by the *eae* genes. Enterohemolysin is encoded by the *hlyA* gene and breaks down the erythrocytes and leads to hemoglobin, which is an iron source for bacteria (Paton and Paton 1998).

Today, studies on the identification of *E. coli* O157: H7 serotypes are continuing all over the world and in parallel to this, researches have been carried out in order to determine the presence of the bacteria in various sources in Turkey. However, this study was conducted, especially considering the fact that isolation of the bacteria from some foods of animal origin in the Hatay region have not been adequately studied. In this study, it was aimed, i) to detect the presence of *E. coli* O157:H7 in food samples (minced meat, Carra and Surk cheeses), ii) to determine some virulence genes, and iii) to evaluate monthly and seasonal distribution of the isolates.

## MATERIALS AND METHODS

*Study area and sampling*

Hatay province, which is located at the south of Turkey and on the eastern Mediterranean coast. Mediterranean climate prevails in Hatay. Summers are hot and dry, while winters are mild and rainy. In this area, the annual average temperature is 18.2ºC. The maximum average temperature is 31.9ºC, in August. The minimum average temperature is 4.7ºC, in January (TSMS, 2017).

A total of 131 food samples (71 Hatay's traditional cheeses (35 Surk, 36 Carra), 60 minced meat) were collected as 5 or 6 samples per month from July 2015 to June 2016. After the samples were brought to the laboratory under cold chain, they were analysed for the presence of *E. coli* O157 and *E. coli* O157:H7 by immunomagnetic separation (IMS) based cultural technique.

*Microbiological analysis*

Twenty-five grams of each minced meat and cheese samples were taken in a sterile bag and pre-enriched with 225 ml of EC broth (Merck110765, Darmstadt, Germany) containing novobiocin (20 mg/l, Oxoid SR0181E, Basingstoke, Hampshire, England) (Cagney et al. 2004, Rey et al. 2006). Then, the samples were incubated at 37°C for 18-24h. After pre-enrichment, IMS based selective enrichment technique was used. IMS was applied as recommended by the manufacturer. The reference strain (*E. coli* O157:H7 ATCC 43888) was used as a positive control.

*IMS method*

For selective separation and concentration of *E. coli* O157, 1ml of the pre-enriched sample aliquot was mixed with 20 µl of magnetic beads coated with

specific antibodies against *E. coli* O157 (Dynabeads anti-*E. coli* O157, cat. no. 71004, Thermo Fisher Scientific, Lithuania). To prevent the beads from settling, the rack was gently and continuously shaken for 10 min. Then, the tubes were allowed to stand for 3 min for the formation of antigen-antibody complex in a magnetic field. The formation of bead-bacteria complex was observed on the sides of the tubes and 100 µl of washing buffer (PBS-Tween) was added to resuspend the beads. After IMS, resuspended beads was plated onto Sorbitol MacConkey Agar (SMAC) (Acumedia, Lansing, Michigan) supplemented with Cefixime-tellurite supplement (CT) (Oxoid SR0172, Basingstoke, Hampshire, England). Plates were incubated at 42°C for 24h.

*Latex agglutination test*

After incubation, colorless colonies (sorbitol negative) on CT-SMAC were evaluated as suspect *E. coli* O157 and subjected to latex agglutination test (*E. coli* O157 Latex, Oxoid DR0620M, Basingstoke, UK) with O157 antigen. Up to five agglutination positive colonies were selected and stored at -20°C for PCR analysis.

*PCR analysis*

For this purpose, commercially available bacterial DNA extraction kit (Nucleic Acid Extraction Kit, GF-1, Vivantis, Malaysia) was used. DNA extraction from the isolates was performed by applying the steps sequentially outlined in the kit. DNAs were stored at -20°C for further analysis.

*Confirmation of the isolates*

To verify the obtained isolates in terms of *E. coli* O157:H7, amplification of the $rfb_{O157}$ and $fliC_{h7}$ genes in PCR assay was targeted (Table 1). For this purpose, ready-to-use master mix (Dream Taq Green PCR Master Mix, 2X, Thermo Scientific K1081, Lithuania) was used. Primers were added to the PCR mix to give a final concentration of 0.50 µM. The total volume was adjusted to be 50 µl with PCR Grade water. A 2 µl aliquot of template DNAs was added to the mix.

For amplification of the $rfb_{O157}$ gene (Maurer et al. 1999), initial denaturation was applied at 94°C for 5 min and then 30 cycles of denaturation at 94°C for 1min, annealing at 53°C for 1min, and extension at 72°C for 1min, with a final extension for 5min at 72°C. Amplification of $fliC_{h7}$ (Sarimehmetoglu et al. 2009) was carried out with the initial denaturation at 94°C for 2min, followed by 35 cycles of denaturation at 94°C for 20s, annealing at 54°C for 1min, and extension at 72°C for 1min, with a final extension for 10min at 72°C.

*Detection of the virulence genes*

The presence of some virulence genes ($stx_1$, $stx_2$, *eaeA*, *hlyA*) were investigated by multiplex PCR in the isolates confirmed as *E. coli* O157:H7. For this purpose, PCR conditions described by Fratamico et al. (2000) and ready-to-use master mix (Dream Taq Green PCR Master Mix) were used. Specific primers (Table 1) for *stx1, stx2,* and *hlyA* genes were added to the PCR mix to give a final concentration of 0.50 µM, while *eaeA* gene-specific primers were used as 0.25 µM. The total volume was adjusted to be 50 µl with PCR Grade water. Template DNAs were added in a volume of 5µl. PCR amplification was performed similarly to the amplification conditions of the $fliCh_7$ gene. Amplification products were subjected to 1.5% agarose gel electrophoresis carried out at 100V for 50min (CS-300V, England). Gene-specific DNA bands were then evaluated under UV light on a gel imaging system (UVP, USA).

**Table 1:** Primer sequences and amplicon sizes used in the study
**Tablo 1:** Çalışmada kullanılan primer sekansları ve amplikon büyüklükleri

| Target gene | Primer sequence (5'-3') | Amplicon size (bp) | Reference |
|---|---|---|---|
| $rfb_{O157}$ | PF8: CGTGATGATGTTGAGTTG<br>PR8: AGATTGGTTGGCATTACTG | 420 | Maurer et al. (1999) |
| $fliC_{h7}$ | FLICH7-F:GCGCTGTCGAGTTCTATCGAGC<br>FLICH7-R: CAACGGTGACTTTATCGCCATTCC | 625 | Schoenhals and Whitfield (1996) |
| $Stx_1$ | SLT1-F: TGTAACTGGAAAGGTGGAGTATACA<br>SLT1-R: GCTATTCTGAGTCAACGAAAAATAAC | 210 | Meng et al. (1997) |
| $Stx_2$ | SLTII-F: GTTTTTCTTCGGTATCCTATTCC<br>SLTII-R: GATGCATCTCTGGTCATTGTATTAC | 484 | Meng et al. (1997) |
| *eaeA* | AE22: ATTACCATCCACACAGACGGT<br>AE20-2: ACAGCGTGGTTGGATCAACCT | 397 | Fratamico and Strobaugh (1998) |
| *hlyA* | MFS1-F: ACGATGTGGTTTATTCTGGA<br>MFS1-R: CTTCACGTCACCATACATAT | 166 | Fratamico and Strobaugh (1998) |

## RESULTS

In this study, 60 minced meat and 71 cheese samples (36 Carra, 35 Surk) were analysed for the presence of *E. coli* O157, *E. coli* O157:H7 during a 1-year period. Overall, *E. coli* O157 was detected in 7 minced meat, and 10 cheese samples. A total of 30 isolates from the 17 positive samples were identified as *E. coli* O157. Among them 11 were recovered from minced meat, and 19 were from cheese samples. All of the 30 isolates were positive for $rfb_{O157}$ by PCR, whereas $fliC_{h7}$ was found in 28 of them. It means that 28 of the isolates were motile and confirmed as *E. coli* O157:H7.

Table 2 presents monthly and seasonal distribution of the isolates obtained in this study. Half of the total isolates (50%) were obtained in spring. During autumn no isolation was observed. In this study, interestingly the prevalence of *E. coli* O157 and *E. coli* O157:H7 was found to be a little greater during summer (8/30, 26.6%) compared to the winter (7/30, 23.3%). Data in Table 2 shows that the isolates were mostly recovered from minced meat samples in May with a rate of 45.4% (5/11) while in cheese samples, isolates were mainly obtained in April and February, at a level of 36.8% (7/19) and 31.5% (6/19), respectively.

**Table 2:** Monthly and seasonal distribution of *E. coli* O157 and *E. coli* O157:H7 isolates
**Tablo 2:** *E. coli* O157 ve *E. coli* O157:H7 izolatlarının aylık ve mevsimsel dağılımı

| Sample | Sampling period | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Winter | | | Spring | | | Summer | | | Autumn | | |
| | Dec. | Jan. | Feb. | Mar. | Apr. | May | June** | July* | Aug | Sept. | Oct. | Nov. |
| Minced meat (*n*=11) | 0 | 1 | 0 | 2 | 0 | 5 | 1 | 2 | 0 | 0 | 0 | 0 |
| Cheese (*n*=19) | 0 | 0 | 6 | 0 | 7 | 1 | 2 | 2 | 1 | 0 | 0 | 0 |
| Total (*n*=30) | | 7 | | | 15 | | | 8 | | | 0 | |

*n,* number of isolates; *, date the study started (2015); **, date the study finished (2016).

Presence of some virulence genes ($stx_1$, $stx_2$, *eaeA,* and *hlyA*) in the isolates was analysed by the multiplex PCR method (Figure 1). No *hlyA* gene was found in any of the isolates. The isolates were found to have the *eaeA* gene at a level of 83.3% (25/30). From minced meat, eight isolates harbouring the *eaeA* gene, while 17 isolates from cheese samples were carrying this gene. Also, $stx_2$ gene was detected in 6.6% (2/30) of the isolates. These two isolates having both $stx_2$ and *eaeA* genes at the same time were obtained from minced meat. There were no genes encoding Stx1 and Stx2 toxins in the isolates obtained from the cheese samples.

When virulence properties of the isolates were compared between warmer and colder months, isolates recovered from minced meat during warm months were potentially pathogenic having both *eaeA* and *stx2* genes. The majority of isolates with virulence characteristics were obtained from cheese samples in colder months while they were recovered from minced meat at most in warmer months (Table 3).

**Table 3:** Virulence genes profiles of the isolates in warmer and colder months
**Tablo 3:** İzolatların sıcak ve soğuk aylardaki virülens gen profilleri

| Sample | Virulence genes | | | | Warm | Cold |
|---|---|---|---|---|---|---|
| | *eaeA* | *hlyA* | $stx_1$ | $stx_2$ | May-October | November-April |
| Minced meat | + | - | - | +/-* | 45.4% ($5^a/8^b/11^c$) | 27.2% ($3^a/3^{b_1}/11^c$) |
| Cheese | + | - | - | - | 26.3% ($5^a/6^b/19^c$) | 63.1% ($12^a/13^{b_1}/19^c$) |

*$stx_2$ gene was not detected in the isolates recovered from minced meat samples during cold months.
[a] number of virulent isolates.
[b] number of isolates obtained from May to October.
[b1] number of isolates obtained from November to April.
[c] total number of isolates.

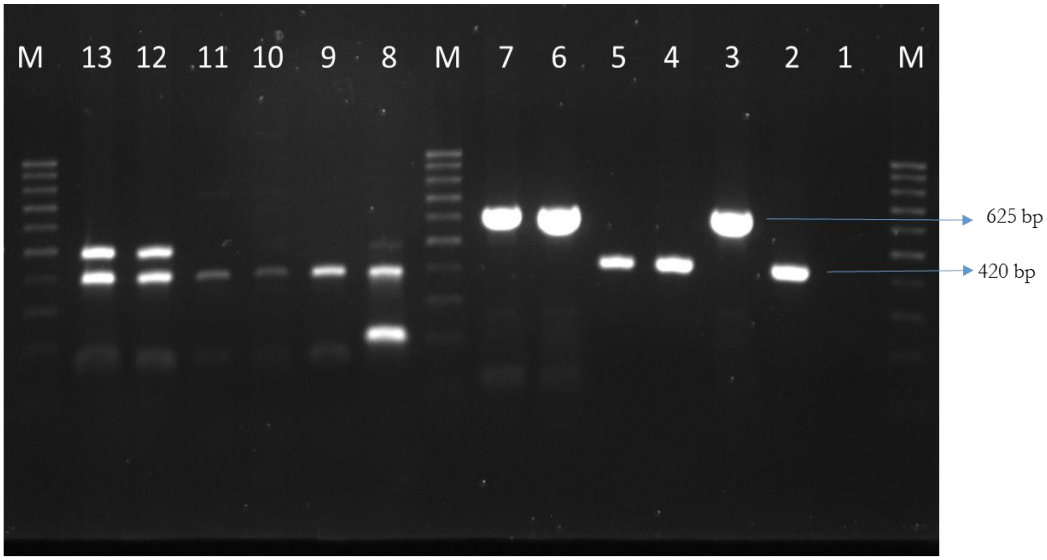


**Figure 1:** Agarose gel image of isolates and their virulence genes. M: Marker, 1: Negative control, 2: Positive control ($rfb_{O157}$), 3: Positive control ($fliC_{h7}$), 4-5: $rfb_{O157}$ positive isolates (420 bp), 6-7: $fliC_{h7}$ positive isolates (625 bp), 8: Positive control [*stx1* (210 bp), *stx2* (484 bp), and *eaeA* (397 bp)], 9-11: *eaeA* positive isolates, 12-13: *eaeA* and *stx2* positive isolates.
**Şekil 1:** İzolatların ve virülens genlerinin agaroz jel görüntüsü. M: Marker, 1: Negatif kontrol, 2: Pozitif kontrol ($rfb_{O157}$), 3: Pozitif kontrol ($fliC_{h7}$), 4-5: $rfb_{O157}$ pozitif izolatlar (420 bp), 6-7: $fliC_{h7}$ pozitif izolatlar (625 bp), 8: Pozitif kontrol [*stx1* (210 bp), *stx2* (484 bp), ve *eaeA* (397 bp)], 9-11: *eaeA* pozitif izolatlar, 12-13: *eaeA* ve *stx2* pozitif izolatlar.

## DISCUSSION

Undercooked minced meat has been responsible for the most of foodborne outbreaks of *E. coli* O157:H7 infections, however, dairy products have been less frequently implicated in the outbreaks (Maurer et al. 1999, Rey et al. 2006, Meng et al. 2007). Although *E. coli* O157:H7 has been commonly detected in beef and beef products, in this study cheese samples were found to be contaminated with *E. coli* O157:H7 at a higher level than minced meat samples.

In this context, *E. coli* O157 and *E. coli* O157:H7 were found to be at the levels of 1-7.6% and 0.79-1%, respectively, in food samples of animal origin (minced meat, raw milk, cheese) in studies carried out in Turkey (Öksüz et al. 2004, Sarimehmetoglu et al. 2009, Çadırcı et al. 2010; Ertas et al., 2013) (Table 4). Abong'o et al. (2009) detected *E. coli* O57:H7 with a rate of 2.8% in meat and meat products in South Africa. However, in this study, the prevalence of *E. coli* O157 and *E. coli* O157:H7 in meat and cheese samples was quite high. In addition, in rectal swab samples in Hatay (Aslantaş et al. 2006) *E. coli* O157 level was found to be quite high as 13.6% compared with other studies conducted in different regions of Turkey. The high level of contamination in Hatay may result from being a border province and its climatic conditions. Similar to this study, *E. coli* O157 and *E. coli* O157:H7 strains isolated from raw meatball (Çadırcı et al. 2010), ground beef (Sarimehmetoglu et al. 2009), and cheese samples (Ertas et al. 2013) had $stx_1$, $stx_2$ and *eaeA* genes.

No research has been conducted on the seasonal distribution of *E. coli* O157 in foods in Turkey, so in this respect our study carries the distinction of being the first. In this study, *E. coli* O57 and *E. coli* O157:H7 were isolated from minced meat in warmer months, whereas they are isolated from cheese in colder months. Surk and Carra, which are analysed in the study, are traditional cheeses produced in Hatay. Especially, Carra cheese is matured by being buried in the soil in a jug after it is produced. It is usually removed from the soil to the January. This means that isolation of *E. coli* O57 and *E. coli* O157:H7 from cheese (especially Carra cheese) has been more found in colder months due to the production technique.

Barkocy-Gallagher et al. (2003) detected *E. coli* O157:H7 at levels of 5.9%, 60.6% and 26.7%, in cattle fecal samples, hide samples, and preevisceration carcass samples, respectively. After cutting, *E. coli* O157:H7 was detected at the level of 1.2% in the carcasses at cooling stage. Also, it has been reported that the prevalence of this pathogen varies seasonally. In this context, it was stated that the contamination with this pathogen reached peak level in summer in fecal samples, while its prevalence in hide samples was high from spring to autumn.

Chapman et al. (1997) isolated *E. coli* O157 with a rate of 15.7%, 2.2% and 0.4% from cattle, sheep, and pig stool samples, respectively, but they could not find it in chickens. The seasonal prevalence of

*E. coli* O157 in cattle was reported to be higher in spring and late summer. Kudva et al. (1997) detected *E. coli* O157:H7 in sheep during the summer months, but they could not find in other seasons. On the contrary, in Scotland, *E. coli* O157 was detected at higher levels in fecal samples of cattle in cooler months than the warmer months. Additionally, 98% of the isolates obtained in winter showed virulence and were found to have the *eaeA*, $vt_1$and/ or $vt_2$ genes (Ogden et al. 2004).

*E. coli* O157 was detected at the highest level in July and November in rectal swab samples of cattle from Hatay, while the lowest level was found in February (Aslantaş et al. 2006). In a study carried out in Kırıkkale, the prevalence of *E. coli* O157 in cattle was found to be a little high in warmer months compared to colder months (Ayaz et al. 2014). Similarly, in this study, the contamination level of food samples with *E. coli* O57 and *E. coli* O157:H7 was faintly higher in summer than winter. In Mexico, 5% and 2.7% of cattle carcasses were contaminated with *E. coli* O157 and *E. coli* O157:H7, respectively. Also, strains isolated from two carcass samples were found to be able to produce shiga-toxin. Regarding the seasonal distribution, the prevalence of *E. coli* O157:H7 was higher in warmer months than colder months (Varela-Hernández et al. 2007).

**Table 4:** Prevalence of *E. coli* O157 and *E. coli* O157:H7 from studies conducted in different provinces of Turkey
**Tablo 4:** Türkiye'nin farklı illerinde yürütülen çalışmalarda *E. coli* O157 ve *E. coli* O157:H7 prevalansı

| Sample | Province | Number of sample | Positive (%) | | References |
|---|---|---|---|---|---|
| | | | *E. coli* O157 | *E. coli* O157:H7 | |
| Raw milk, raw- milk cheese | Tekirdağ | 150 | 2 | - | Oksuz et al. (2004) |
| Ground beef | Ankara | 251 | 7.6 | 0.79 | Sarımehmetoglu et al. (2009) |
| Ground beef, raw meatball | Samsun | 200 | 2.5 | - | Çadırcı et al. (2010) |
| Diced meat, minced meat, burger, raw milk, raw-milk cheese | Kayseri | 500 | 1 | 1 | Ertas et al. (2013) |
| Rectal swab samples from cattle | Hatay | 565 | 13.6 | - | Aslantaş et al. (2006) |
| Feces, colon tissue samples from cattle and sheep | - | 500 | - | 5 | Goncuoglu et al. (2010) |
| Feces from water buffaloes | Afyonkarahisar | 300 | - | 3.6 | Şeker et al. (2010) |
| Rectoanal mucosal swab (RAMS), carcass sponge, bile samples from cattle and wastewater samples from slaughterhouse | Kırıkkale | 240 cattle | 7.1 (RAMS or carcass sponge) | 6.3 (RAMS or carcass sponge) | Ayaz et al. (2014) |
| | | 24 wastewater | 20.8 (wastewater samples) | - | |

## CONCLUSIONS

Our results show that high level of contamination with *E. coli* O157:H7 in food samples. Also, strains obtained from minced meat having ability to produce shiga-like toxins and this appears to be a problem within the scope of human infections. So that, hygienic measures should be taken in the production of traditional cheeses and minced meat in Hatay.

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