شناسایی و اختصاصات سویه‌های تولیدکنندهٔ سم شیگا *اشریشیا کلی* جداشده از سطوح در تماس با گوشت

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چکیده

مقدمه: سروتیپ‌های تولیدکنندهٔ سم شیگا باکتری *اشریشیا کلی* جزو مهم‌ترین عوامل نوپدید بیماری‌های مشترک انسان و حیوان به شمار می‌آیند که سبب بیماری‌هایی مانند کولیک خونریزی‌دهنده، سندرم اورمی‌همولیتیک و ترومبوسایتوپنیای ترومبوتیک در انسان می‌شوند. مطالعهٔ حاضر با هدف تعیین آلودگی سطوح مرتبط با گوشت به *اشریشیا کلی* O157:H7 و تعیین حضور ژن‌های حدت در سروتیپ‌های جداشده به روش PCR اجرا شد.

مواد و روش‌ها: در مطالعهٔ حاضر، تعداد ۱۱۱ نمونه از کشتارگاه‌ها و مراکز عرضه‌کنندهٔ گوشت دریافت شدند که تعداد ۵۵ نمونه (۴۹/۵۴ درصد) به کمک آزمون‌های تشخیصی، *اشریشیا کلی* تشخیص داده شدند؛ سپس جدایه‌های تأییدشده برای بررسی وجود *اشریشیا کلی* O157:H7 و تعیین حضور ژن‌های حدت (*Stx1*، *Stx2*، *eae* و *Hly*) به روش PCR بررسی شدند.

نتایج: باکتری *E.coli* O157 تنها در ۱۴ نمونهٔ (۱۲/۶۱ درصد) متعلق به کشتارگاه و از این تعداد، سروتیپ *E.coli* O157 :H7 تنها در ۲ جدایه (۱/۸ درصد) تشخیص داده شد. پس‌از انجام PCR مشخص شد تعداد ۴ جدایه حاوی ژن‌های *Stx1*، *Stx2* و *Hly*، ۲ جدایه حاوی ژن‌های *Stx1*، *eae* و *Hly*، ۳ جدایه حاوی ژن‌های *Stx1* و *Hly*، ۱ جدایه حاوی ژن‌های *Stx2* و *eae* و ۳ جدایه حاوی ژن *Hly* هستند و ۱ جدایه هیچ‌کدام از ژن‌ها را ندارد.

بحث و نتیجه‌گیری: بر اساس نتایج مطالعهٔ حاضر، محیط کشتارگاه و سطوح مرتبط با گوشت منابع مهمی برای آلودگی لاشه‌ها به سروتیپ O157: H7 باکتری *اشریشیا کلی* و به‌خطرافتادن مصرف‌کنندگان هستند؛ ازاین‌رو، انجام اقدام‌های بهداشتی و ضدعفونی در کشتارگاه‌ها به‌شدت توصیه می‌شود.

واژه‌های کلیدی: *اشریشیا کلی*، شیگاتوکسین، سطوح در تماس با گوشت، ژن‌های حدت

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Identification and Characterizations of Shiga Toxin-producing *E.coli* Isolated from Meat-contact Surfaces

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Abstract

Introduction: Shiga Toxin-producing *Escherichia coli* (STEC) is an important organism known as an emerging zoonotic microorganism causing diseases such as hemorrhagic colitis and Hemolytic Uremic Syndrome (HUS) as well as thrombotic thrombocytopenia in humans. This study aimed to determine the contamination of meat-contact surfaces to STEC and the characterization of the virulence genes of the isolates.

Materials and Methods: Totally, 111 swab samples were obtained from meat-contact surfaces in slaughterhouses and meat supply centers for 6 months. After the primary enrichment and cultivation on EMB and SMAC environments, sorbitol negative colonies were transferred to differential media and confirmed by specific tests as *Escherichia coli*. Suspected colonies were evaluated by PCR method to determine the existence of serotype O_{157}: H_7 and virulence genes such as *Stx1*, *Stx2*, *eae*, and *Hly*.

Results: *E. coli* O_{157} was detected in 14 samples (12.61%), and only 2 isolates (1.8%) were identified as *E.coli* O_{157}: $H_{7.}$ In PCR, 4 isolates contained *Stx1*, *Stx2*, and *Hly* genes, 2 isolates contained *Stx1*, *eae* and *Hly* genes, 3 isolates contained *Stx1* and *Hly* genes, 1 isolate contained *Stx2* and *eae* genes, 3 isolates contained the *Hly* gene and 1 isolate did not have any of the virulence genes.

Discussion and Conclusion: Concerning the possibility of the transmission of pathogens such as *E.coli* O_{157}: H_7 from contaminated surfaces to carcasses and healthy meat, the lack of attention to the health and care of slaughterhouses and meat supply centers can be concerns for the public health.

Keywords: *E. coli*, Shiga Toxin, Meat-contact Surfaces, Virulence Gene.

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Introduction

Escherichia coli, a member of the Enterobacteriaceae family, is a facultative anaerobic, gram-negative, small bacilli. This bacterium is one of the predominant species in the human digestive system, whose pathogenic forms can cause various forms of diarrhea. This variation depends on its pathogenic factors and genes (1). There are currently six pathogens of *Escherichia coli*, including Enterotoxigenic *E.coli* (ETEC), Enteroinvasive *E.coli* (EIEC), Enteropathogenic *E.coli* (EPEC), Enterohemorrhagic *E.coli* (EHEC), Enteroaggregative *E.coli* (EAgEC) and highly adherent *E.coli* (DAEC) (2). *Escherichia coli* O_{157}: H_7 is the most important serotype in the EHEC group and plays a crucial role in the occurrence of diseases such as hemorrhagic colitis, hemolytic uremic syndrome, and idiopathic purpura thrombocytopenia (3). This serotype is known as a strain of Verotoxigenic *E.coli* causing human infection in many parts of the world such as Europe, America, and Asia (4, 5). Most cases of hemorrhagic colitis and hemolytic uremic syndrome are attributed to STEC O_{157} strains (6). The contamination with this strain is often due to the consumption of minced meat. The origin of contamination of minced meat is related to carcass contamination because cows can carry bacteria in their stool and cause the contamination of the skin and carcasses (7). Although the transmission of *E. coli* O_{157}: H_7 is mainly by the consumption of beef-based foods, in recent years, various types of foods such as goat milk, lettuce, apple juice, and alfalfa buds have also been identified as foods which cause the transmission of this organism (8-11). Other transmission ways of this organism are from person to children in care centers, and swimming in water which is contaminated with feces (12). The infectious dose of *E. coli* O_{157}: H_7 is very low like Shigella so that less than 100 bacteria are enough to cause the disease. Indeed, the age and quality of the host immune system are very effective in the disease process; the bacterial resistance to the acidic environment is one of the causes of the low infectious doses of the strain and an infusion dose of 0.3-15 cfu/g has been calculated in the hamburgers which cause food infections (13, 14). STEC strains are known by the production of one or more types of Shiga toxins (Stx1, Stx2). The function of these toxins is to prevent protein production in the host cell and cause cell killing. These toxins are produced by EHEC and based on the ability to produce the toxins are called Verotoxigenic *E. coli* (VETEC) or Shiga Toxin-producing *E. coli* (STECs). These strains produce two types of toxins that are related to the toxins which are produced by the *Shigella disantrea* Type 1 (15). Most strains of STEC are human pathogens and contain *eae* gene, the *eae* gene codes for an extracellular protein called intimin. This protein is essential for binding bacteria to host enterocytes. Other pathogenicity factors include hemolysin which acts as a cytolysin on eukaryotic cells and is coded by the *Hly* gene (6, 16). Hence, the current study was conducted to investigate the contamination of meat-related surfaces in production centers and supply of protein products in Chaharmahal and Bakhtiari province to *E. coli* O_{157}: H_7 and also to determine the presence of *Stx1*, *Stx2*, *eae* and *Hly* genes in isolated serotypes by PCR.

Materials and Methods:

In this study, 111 samples of meat

contact surfaces were obtained from slaughterhouses and meat supply centers in Chaharmahal and Bakhtiari province. Samples were taken by 100 cm² sterile wet swabs and the meat-related surfaces per unit were repeated three times. The swab was placed in *E. coli* broth (Merck, Germany) and incubated at 37 ^{o} C for 18-24 hours, then 100 microliters of the enriched sample were cultured on Sorbitol McA (Merck, Germany) and Eosin methylene blue (Merck, Germany) containing cefixime and potassium tellurite. Plates were incubated for 24 hours at 37 °C. Sorbitol-negative colonies were examined by IMVIC test to confirm the *E. coli* (17). Isolated *E. coli* were tested using *rfbE* and *H7* genes to confirm the *E. coli* O_{157}: H_7 and the existence of *Stx1*, *Stx2*, *eae*, and *Hly* genes by PCR (Table 1) (18-21).

To identify *rfbE* and *H7* genes, Multiplex PCR and, for other genes single PCR were performed. The master mix (CinnaGene-Iran) used in the PCR reaction in a volume of 20 µl, including 2.5 µl 10-fold PCR buffer, 1 µl $MgCl_2$ 50 mM, 1.5 µl primer, 0.15 µm Taq DNA polymerase and 2 µl of DNA template. PCR was performed in 35 cycles using a thermocycler (Biorad, USA), (Table 2). The PCR product was electrophoresed on 1% agarose gel using 95 volts for 45 minutes.

Table 1- The Primers Used for the Identification of *E.coli* O157: H7 and Virulence Genes[18-21]

Target gene	Primers	DNA primer sequence ('3-'5)	Length bp	Annealing temperature	References
rfbE	*O157*-R *O157*-F	CGT GGT ATA GCT ACT GTC ACC CGC TGA ATG TCA TTC GCT CTG C	259	58	Paton, A.W., Paton, J.C., 1998
Stx1	*Stx1*-F *Stx1*-R	ATA AAT CGC CAT TCG TTG ACT AC AGA ACG CCC ACT GAG ATC ATC	180	48	Paton, A.W., Paton, J.C., 1998
Stx2	*Stx2*-F *Stx2*-R	TTA ACC ACA CCC CAC CGG GCA GT GGA TAT TCT CCC CAC TCT GAC ACC	524	55	Pollard DR, Johnson WM, Tyler SD et al. ,1990
eae A	*Eae*-R *Eae*-F	GCG GTA TCT TTC GCG TAA TCG CC GAG AAT GAA ATA GAA GTC GT	775	50	Reid SD, Betting DJ, Whittam TS , 1999
H7	*H7*-R *H7*-F	CAA CGG TGA CTT TAT CGC CAT TCC GCG CTG TCG AGT TCT ATC GAG C	625	60	Gannon, V.P., et al. ,1997
Hly A	*Hly*-R *Hly*-F	TGG GCT GGA TGT TGT CTC C CCA TCA TCG CCG TAT AGT CG	513	59	Accession no: AP018489.1

Table 2- The Applied Thermal Cycles to Perform PCR

Cycles	Temperature ^{o}C	Time	Numbers
Initiation	95	4 min	1
Denaturation Annealing Extension	95	30 s	35
	58 (*rfbE* , *H7*) Variable for other genes	40 s	
	72	60 s	
Final Extension	72	5 min	1

Results

A total of 84 samples from slaughterhouses and 27 samples from meat supply centers were collected, 55 isolates (49.54%) were identified as *E. coli* by microbiological and biochemical tests. Also, the results indicated that 14 (25.5%) of the isolated *E.coli* were O157 serotype, of which 2 (3.6%) of them were confirmed as *E. coli* O_{157}: H_7 (Fig. 1).

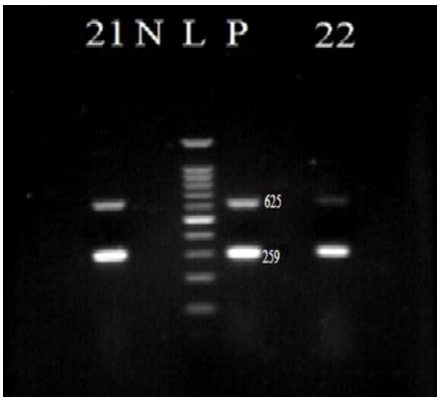

Fig. 1- Identification of the *rfbE* and H_7 Genes by the Multiplex PCR. (The expected band for *rfbE* gene: 259 and for H7: 625 gene). L: Ladder, P: Positive Control, N: Negative Control, H7 Positive Samples: 21 and 22

The existence of *Stx1*, *Stx2*, *eae*, and *Hly* genes were evaluated in 14 isolates of *E. coli* O157 and O157:H7; the results were as follows: 4 isolates (28.57%) contained *Stx1*, *Stx2*, and *Hly* genes, 2 isolates (14%) contained *Stx1*, *eae* and *Hly* genes, 3 isolates (21.42%) contained *Stx1* and *Hly* genes, 1 isolate (7.21%) contained *Stx2* and *eae* genes, 3 isolates (21.42%) contained *Hly* genes, and 1 isolate (7.21%) did not have any of the genes (Tables 3 and 4) (Figs. 2-5).

Table 3- *Escherichia coli* Strains Isolated from Meat-contact Surfaces

Sampling site		Numbers	*E.coli*	O_{157}	O_{157}: H_7
Slaughterhouse	Livestock slaughterhouse	60	42 (70%)	14 (33%)	2 (4.7%)
	Chicken slaughterhouse	24	5 (20.8%)	0	0
Meat supply centers	Meat	12	1 (8.3%)	0	0
	Chicken	15	7 (46.6%)	0	0

Table 4- Frequency of Virulence Genes in Isolated *E. coli* O_{157} and *E. coli* O_{157}: H7

Serotype	Numbers	Virulence genes			
		Stx1	*Stx2*	*eae*	*Hly*
E.coli O_{157}	12	7 (57.33%)	3 (25%)	3 (25%)	10 (83.33%)
E.coli O_{157}:H_7	2	2 (100%)	2 (100%)	2 (100%)	2 (100%)
Total	14	9 (67.3%)	5 (35.7%)	5 (35.7%)	12 (85.7%)

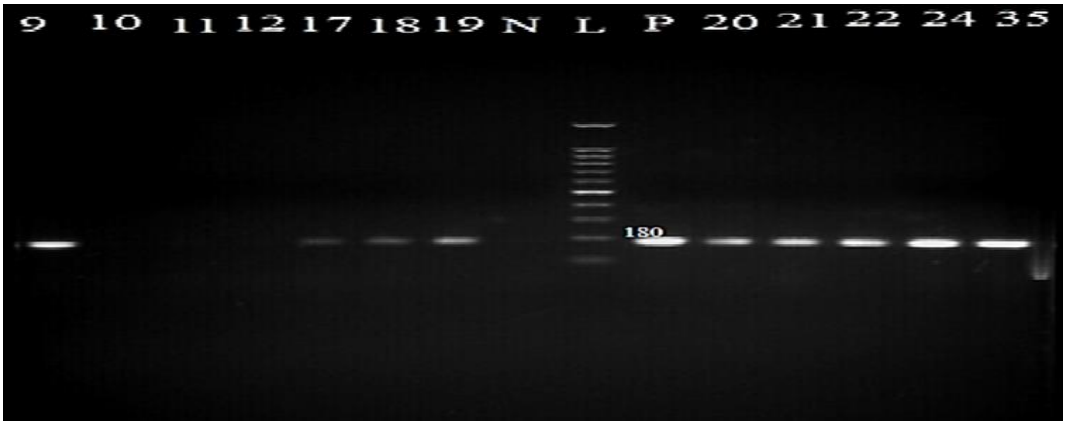

Fig. 2- Identification of the *Stx1* Gene (Expected Band 180) L: Ladder, P: Positive Control, N: Negative Control, *Positive Samples:* 9, 17, 18, 19, 20, 21, 22, 24, 35

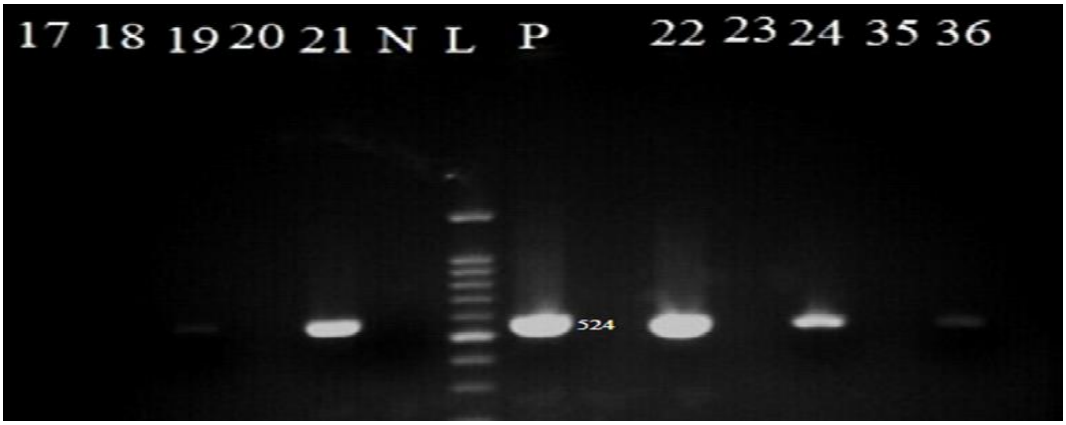

Fig. 3- Identification of the *Stx2* (Expected Band: 524) L: Ladder, P: Positive Control, N: Negative Control, *Positive Samples: 19, 21, 22, 24, 36*

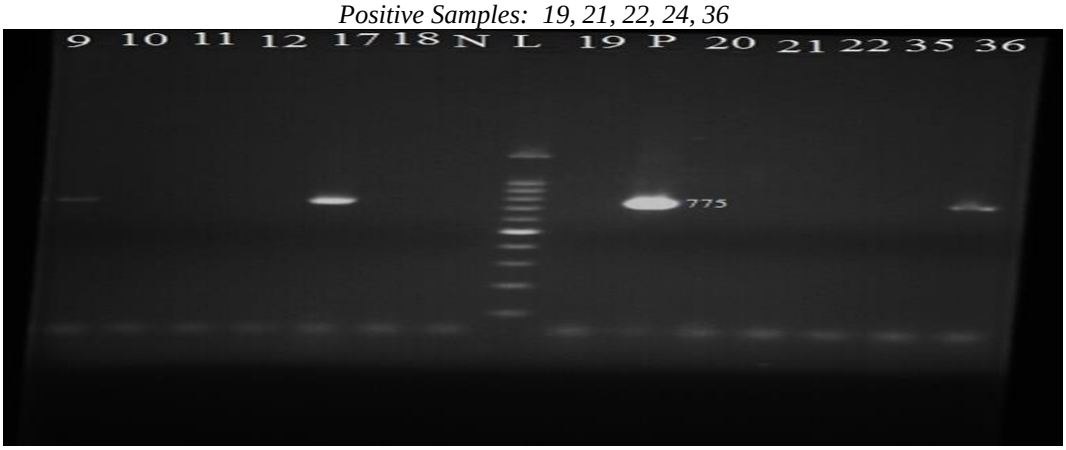

Fig. 4- Identification of the *eae* Gene (Expected Band: 775) L: Ladder, P: Positive Control, N: Negative Control, *Positive Samples:* 9, 17, 36

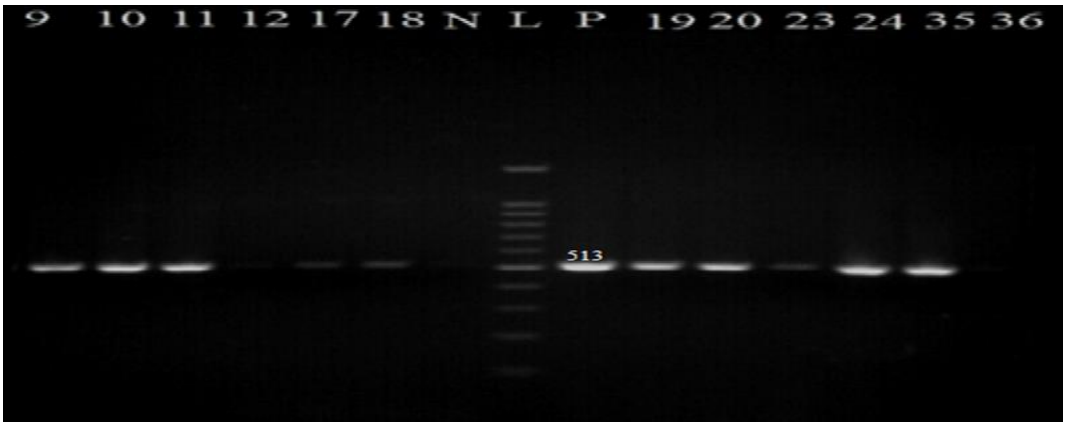

Fig. 5- Identification of the *Hly* Gene (Expected Band: 513). L: Ladder, P: Positive Control, N: Negative Control, *Positive Samples:* 9, 10, 11, 17, 18, 19, 20, 23, 24, 35

Discussion

E. coli O_{157}: H_7 is an emerging foodborne pathogen that can cause significant human diseases. Food or water contamination with feces of ruminants especially cattle and goat are considered as the primary reservoirs of STEC strains. The serotype O_{157}: H_7 is the main cause of human diseases (22). Most cases of hemorrhagic colitis and hemolytic uremic syndrome are attributed to STEC O_{157} strains (6).

According to the results of this study, the

meat-contact surfaces contamination with the *E. coli* O_{157}: H_7 was 1.8%, but other verotoxigenic strains were more prevalent. Also, the results revealed that all of the *E. coli* O_{157} strains were related to livestock slaughterhouse samples. These results confirmed that the livestock especially cattle were the main reservoirs of the O_{157} strain. Dolye and Schoeni in the United States showed that from 205 sheep samples, 4 samples (1.5%) were contaminated with *E. coli* $O_{157:}$ H_7 (23). A study by Hiko et al. from Ethiopia showed that 2.5% and 2% of sheep and goats were contaminated with *E. coli* O_{157}: H_7, respectively (24). The results of these two studies and the present study indicate that this serotype is not prevalent. Jafaryan et al. reported that the prevalence of sheep contamination to *E. coli* O_{157}: H_7 was 3.4% (25). Similarly, Shekarforoush et al. reported that the prevalence of sheep contamination with *E. coli* O_{157}: H_7 in Shiraz was 3.9% (26). Also, Phillips et al. in Australia reported that the contamination rate of sheep meat to *E. coli* O_{157}: H_7 was 0.5% (27). In Italy, the sheep contamination to *E. coli* O157: H7 was less than 1% (0.77%) but, another study in this country by Franco et al., using the immuno-magnetism technique, showed that the sheep contamination rate was 7.1% (28, 29).

Dontorou et al. in Greece, using microbiological, serology, and PCR methods showed that out of 351 feces of sheep, goat, and cattle only 1 sample of goat stool (0.2%) carried this pathogen (30). Johnsen et al. in Norway showed that 7.4% of the sheep's stool specimens were contaminated with *E. coli* O_{157}: H_7 (31). Studies revealed a significant relationship between the prevalence of *E. coli* O_{157}: H_7 in feces and carcass contamination (32).

Comparing these results with the result of the present study showed that although the level of contamination of the meat-related surface with *E. coli* O_{157}: H_7 was not high, there was a risk of transmission of this bacterium from contaminated surfaces to healthy meat and human, especially when the isolates harbored the virulence genes. The level of contamination of meat with STEC harboring virulence genes has been investigated by various researchers in the world. In a study in Ireland, out of 905 samples (minced meat, carcasses, and stools of cattle and sheep), 65 samples were contaminated with *E. coli* O_{157}. Of these, 41 strains had *Stx2* and *eae* genes, and 4 strains had *Stx2*, *Stx1*, and *eae* genes (33). Also, Bonyadian et al. in Shahrekord, Iran, showed that the rate of contamination of minced meat and hamburger to *E. coli* O_{157} was 3.2%, but none of them were serotype O_{157}: H_7. Also, the results showed that three isolates (37.7%) had *Stx2* and *eae* genes, two isolates were related to minced meat and one isolate was related to hamburger; however, none of the isolates possessed *Stx1* and *Hly* genes (34). Kargar at el. in Shiraz, reported three confirmed serotype of O_{157}: H_7 isolated from raw milk, two strains harbored *Stx1* and *eae* genes (35). Of the 22 strains of STEC isolated in Mexico, most strains either had the *Stx2* gene or the combination of the *Stx1* and *eae* genes (36). In the United States, 57 strains of *E. coli* O_{157}: H_7 were isolated from cattle, 38 strains contained *Stx1*, *Stx2*, *eae* and *Hly* genes, 11 strains had *Stx1*, *eae* and *Hly* genes, 5 strains had *Stx2* genes, *eae* and *Hly* and 3 strains had *Stx1*, *Stx2*, and *eae* genes (37).

In the present study, the frequency of *Stx2* and *eae* genes was significantly lower than those of Prendergast et al. in Ireland and Bonyadian et al. in Shahrekord, although the frequency of *Stx1* and *Hly* genes was greater than those of the two studies. The frequency of *Stx1*, *eae*, and *Hly* genes in this study was consistent with the study by Byrne et al. and was more than the results of Garcia's study. Some strains of *E. coli* O_{157}: H_7 did not have any virulence genes, and rarely some strains contained all of the virulence genes. Strains that had

multiple genes were considered to be more pathogenic strains. Studies have shown that the maximum excretion of *E. coli* O_{157}: H_7 occurred through feces during the summer and early autumn and varies from 0 to 61% in dairy farms and fields. The data showed that the carriage of O_{157}: H_7 serotype in cattle had a common relationship with seasonal variation and human disease prevalence (38).

Conclusion

The results of the current study showed that the level of contamination of meat-contact surfaces with *E. coli* O_{157}: H_7 was not high, but the existence of other verotoxigenic strains containing virulence genes, and the transfer of these strains to meat products may cause a risk for the consumers' health.

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Conflict of Interests

The authors had no conflict of interest in this study.

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