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ORIGINAL ARTICLE

# Virulence genes and pulsed- field gel electrophoresis profiles of Shiga toxin-producing *Escherichia coli* isolated from different food samples and patients with acute diarrhea

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## ABSTRACT

**Background and Objectives:** *Escherichia coli* O157: H7 is one of the most important causes of hemorrhagic colitis, and hemolytic uremic syndrome. The present study aimed to isolate *E. coli* O157: H7 from foods and patients with hemorrhagic colitis, and identify Shiga toxin genes, phylogenetic comparison, and antibiotic resistance of the isolates.

**Materials and Methods:** In total 400 samples, including patients stool and food were taken in Isfahan-Iran province. Phenotypic tests and PCR were performed to identify Shiga toxin-producing *E. coli*. The isolated strains were compared phylogenetically by PFGE. Agar disk diffusion was performed to identify the antibiotic resistance of the isolates.

**Results:** Totally, 5 isolates of fecal samples were *E. coli* O157, but only 2 isolates carried H7 gene. Also, 9 isolates of *E. coli* O157 were isolated from food samples that 3 isolates were *E. coli* O157: H7. The isolates carried *stx1*, *stx2*, *hlyA* and *eaeA* genes. Also, *E. coli* non-O157: H7 identified from samples that contained *stx1*, *stx2*, *hlyA* genes. The highest susceptibility to imipenem and the highest resistance to ampicillin and ciprofloxacin were observed. There was a similarity of 100% between the *E. coli* O157: H7 strains isolated from patients and raw milk and minced beef samples.

**Conclusion:** Serotypes other than the O157 of *E. coli* are more prevalent in patients and food. The *E. coli* O157: H7 isolates from patients had 100% genetic similarity with minced meat and cow milk isolates, which indicates cattle are the most important reservoir of this bacterium in Iran.

**Keywords:** Food; Human; Shiga toxin-producing *E. coli*; Pulsed-field gel electrophoresis; Antibiotic resistance

## INTRODUCTION

Increasing food safety promotes community health, and is a health priority. Based on notification of World Health Organization (WHO), many diarrhea agents are transmitted by food (1). Diarrhea is the second cause of death in children under 5 years old. Estimates indicate that 1,700 million cases of diarrhea and 5 million deaths occur among children each year (2). *E. coli* O157: H7 is a common pathogen that contaminates food (3), and transmits the infection through fecal-oral (4), and infective dose in human is less than 4-24 organisms (3, 5). Cattle, are known to be the main reservoir of infection (6). Other ruminants such as goats, sheep (7, 8), and buffalo except camels can also be sources of this pathogen

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(9). Non-ruminants such as pigs and pigeons, have been reported as carriers of this pathogen (8). Fish contamination with *E. coli* O157: H7 may occur through contaminated waters (10). Generally, 100 CFU of bacteria is enough to infect cattle (7), and an oral dose of $10^5$ CFU can also infect sheep (9). Ingestion of contaminated foods or waters can lead to human infection (3). Contaminated foods such as beef and milk (11-13), goat and sheep meats, also, chicken meat may cause human infection (11). Seafood contamination should also be considered because the pathogen has also been isolated from oysters (13). Fast foods may contaminated by this pathogen and cause infection in human (14). The pathogenicity of the bacterium is related to producing Shiga toxin and its intimin protein. Shiga toxin is associated with Hemolytic Uremic Syndrome (HUS), Hemorrhagic Colitis (HC), and dysentery (15, 16). It has been shown that the pathogenicity of Stx2 is more important than Stx1 (17). Excessive and inappropriate use of antibiotics to treat infectious diseases has increased resistance to pathogenic bacteria (18). The WHO warns that resistance to antibiotics is currently a serious concern to public health (19). Increased threat to multiple antibiotic resistances in *E. coli*, especially the third generation of cephalosporin and especially colistin has recently been reported (20). Although the presence of all pathogenic genes studied in one strain can be a reason for pathogenicity, the similarity of virulence factors in isolated strains of different regions can indicate the prevalence of one strain in the study site and a measure of the severity of pathogenicity. The genomic DNA restriction pattern by pulsed-field gel electrophoresis is a useful tool for epidemiologic typing and determining the genetic relatedness of food-borne pathogens (21).

The present study aimed to determine the virulence genes, genetic similarity, and antibiotic resistance of *E. coli* O157: H7 strains isolated from patients and food.

## MATERIALS AND METHODS

**Sampling.** One hundred samples (38 males and 62 females) of feces of patients with dysentery from 7 hospitals and clinical laboratories, and 300 food samples including 100 samples of minced meat, 100 samples of chicken meat, and 100 samples of raw milk over 12 months in the city of Isfahan were taken.

**Isolation of *E. coli* O158: H7.** To isolate *E. coli* O157: H7, the samples were inoculated in Escherichia broth (EC) (Oxoid) and tryptone soy broth (Difco) with 20 mg / L novobiocin (Sigma) and incubated for 24 hours at 37°C. Sorbitol-MacConkey and Macconkey agar (Merck, Germany) were used to detect and isolate enterohemorrhagic *E. coli* (EHEC) colonies. Biochemical tests including oxidase, Gram staining, IMViC, urease, and $H_2S$ production were performed to confirm *E. coli.* Bacteriological tests were also performed on non-fermenting lactose colonies to differentiate the main causes of diarrhea such as Salmonella and Shigella (22, 23).

**DNA extraction and PCR assay.** One ml of bacterial culture was poured into 1.5 ml tube containing tryptone soy broth and centrifuged at 12000 rpm for 5 minutes. The supernatant was discarded, and precipitate mixed with 400 µL of sterile distilled water. The suspension was placed in boiling water at 100°C for 5 minutes. The tubes were centrifuged for 5 minutes, and the supernatant DNA concentrations were measured by spectrophotometer at 263 nm. Suspected colonies were examined by PCR using two primers, rfbE (O157) and H7, to confirm *E. coli* O157: H7. Also, specific primers were used to identify *stx1, stx2, hlyA,* and *eaeA* genes (Table 1), (24). The PCR reaction was performed at 25 µL as follows: PCR Master Mix (Sinagen-Iran) 21 µL, each primer 1 µL, and 2 µL of extracted DNA were used (24). Thermal cycles were performed by a thermocycler (Bio-Rad, USA) according to the program (Table 2).

PCR products were electrophoresed using 0.5% agarose in 0.5% buffer TBE at 100 volts for 50 minutes. The gels were stained safe red and photographed with UV light using the gel dock (Herolab, Weisloch, Germany) (24).

**Pulsed Field Gel Electrophoresis (PFGE).** The CDC guideline was used to perform for Pulsed Field Gel Electrophoresis (25). The bacterial cells were lysed with the proteinase K and plug molds were prepared with low-melting point agarose. The plugs were stored in the presence of the XbaI enzyme at 37°C for 4 hours. The 1% agarose gel and 0.5% Tris-borate-EDTA buffer were used for electrophoresis. Electrophoresis was conducted using a CHEF-DRII (Bio-Rad, Japan) as follows: voltage of 6 v/cm, initial switch time of 2.2 Sec, final switch time of 54.2 Sec, and run time of 19 h. The gel was then stained and photo-

**Table 1.** Primers using for detection of virulence genes of *E. coli* (24).

| Target Gene | Primers | Oligonucleotide Sequence (5'-3') | Product length (bp) | Annealing Temperature | Reference |
|---|---|---|---|---|---|
| *rfbE* | O157-R | CGTGGTATAGCTACTGTCACC | 259 | 58 | (24) |
| | O157-F | CGCTGAATGTCATTCGCTCTGC | | | |
| *stx1* | *stx1*-F | ATA AAT CGC CAT TCG TTG ACT AC | 180 | 48 | (24) |
| | *stx1*-R | AGA ACG CCC ACT GAG ATC ATC | | | |
| *stx2* | *stx2*-F | TTA ACC ACA CCC CAC CGG GCA GT | 524 | 55 | (24) |
| | *stx2*-R | GGA TAT TCT CCC CAC TCT GAC ACC | | | |
| *eaeA* | EAE-R | GCGGTATCTTTCGCGTAATCGC C | 775 | 50 | (24) |
| | EAE-F | GAGAATGAAATAGAAGTCG T | | | |
| *flIc H7* | H7-R | CAACGGTGACTTTATCGCCATTCC | 625 | 60 | (24) |
| | H7-F | GCGCTGTCGAGTTCTATCGAGC | | | |
| *hlyA* | hlyA-F | CATCGGCTGTTATGCTGG | 513 | 56 | Accession number: |
| | hlyA-R | CATCCCAATGTTGCTGGG | | | AP01848901 |

**Table 2.** PCR conditions for amplification of the target genes

| No | Step | *rfbE(O157)* | *H7(flic)* | *hly* and *eae* | *stx1* and *stx2* |
|---|---|---|---|---|---|
| 1 | Initial denaturation | 94ºC/4 min. | 94ºC/4 min. | 94ºC/4 min. | 94ºC/4 min. |
| 2 | Denaturation | 94ºC /30 sec | 92ºC /30 sec | 94ºC/45 sec. | 94ºC/30 sec. |
| 3 | Annealing | 58ºC/45 sec. | 62ºC/50 sec. | 54ºC/1 min. | 50ºC/30 sec. |
| 4 | Extension | 72ºC/45 sec. | 72ºC/45 sec. | 72ºC/45 sec. | 72ºC/45 sec. |
| 5 | Final extension | 72ºC/45 sec. | 72ºC/45 sec. | 72ºC/45 sec. | 72ºC/45 sec. |

graphed, and analyzed by molecular analyst software.

**Antimicrobial susceptibility test.** Brain Heart Infusion Broth (BHI, Oxoid) was used for initial culture of isolates, and the concentration equal to 0.5 McFarland was prepared. The guideline of the Clinical & Laboratory Standards Institute was performed for antibiogram using Muller-Hinton Agar (Oxoid) (26). Antibiotics used include: ampicillin (AM, 10 µg), kanamycin (KAN, 30 µg), trimethoprim-sulfamethoxazole (STX, 25 µg), gentamicin (GM, 10 µg), nitrofurantoin (NIT, 300 µg), ciprofloxacin (CIPRS, 5 µg), chloramphenicol (CLR, 30 µg), nalidixic acid (NAL, 30 µg), ceftazidime (CAZ, 30 µg), and imipenem (IPM, 10 µg).

## RESULTS

Shiga-like toxin-producing *Escherichia coli* strains isolated from stool and food samples. The rate of fecal contamination with *E. coli* O157: H7 was 2%, O157: HN was 3%, and non-O157 was 6%. In raw milk, the contamination rates of these strains were 1%, zero, and 3%, in chicken samples zero, 1%, and 3%, and in minced beef 2%, 3%, and zero, respectively (Table 3, Figs. 1 and 2).

Table 4 shows the prevalence of virulence genes among isolated *E. coli*, as the results show the *E. coli* O157: H7 isolates from feces, and different foods containing *stx1*, *stx2*, *hlyA*, and *eaeA* genes. Also, the isolates other than O157 serotypes harbored *stx1*, *stx2*, and *hlyA* genes (Figs. 3-6).

According to the results of antibiotic resistance tests, 24 STEC strains isolated from patients and various foods were sensitive to imipenem. While isolates from food showed the highest resistance to nalidixic acid (76.9%) and ampicillin (69.21%) in this study, isolates from patients were highly resistant to ciprofloxacin and ampicillin (54.5%) (Table 5).

Based on the results of PFGE, the *E. coli* O157: H7 isolates were placed in 3 clusters. Cluster A includes 2 strains (270F and 58H) isolated from meat and a patient with 100% similarity. Also, there is cluster B that includes 2 strains (121F and 36H) with 100% similarity, isolated from raw milk and a patient. Clus-

**Table 3.** Prevalence of *E. coli* isolated from diarrheal and food samples.

| Samples | No | Shiga-like-producing *E. coli* isolates (%) | | | | |
|---|---|---|---|---|---|---|
| | | *E. coli* | *E. coli* O157 | *E. coli* O157: H7 | *E. coli* Non O157 | Total |
| Feces | 100 | 78 | 5 | 2 | 6 | 11 |
| Raw milk | 100 | 66 | 4 | 1 | 0 | 4 |
| Chicken | 100 | 84 | 3 | 0 | 1 | 4 |
| Minced Meat | 100 | 71 | 2 | 2 | 3 | 5 |
| Total | 400 | 75.74 | 3.5 | 1.25 | 2.5 | 6 |

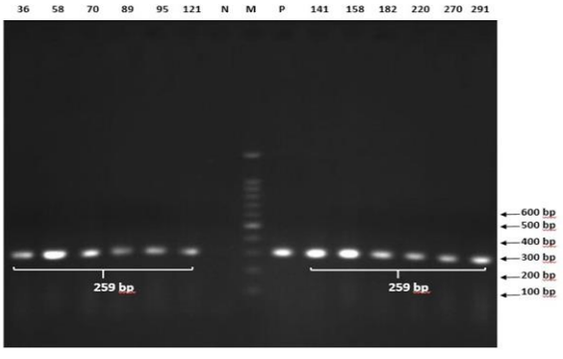


**Fig. 1.** PCR products for the O157 *rfbE* gene. M: 100bp DNA ladder,
N (Negative control), P (Positive control).

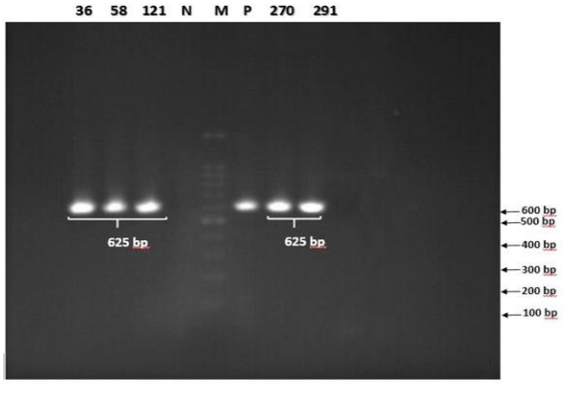


**Fig. 2.** PCR products for the *h7* gene. M: 100 bp DNA ladder,
N (Negative control), P (Positive control).

ter C includes a strain isolated from minced meat (291F) with a genetic similarity of less than 50% with other isolates (Fig. 7).

## DISCUSSION

Shiga toxin-producing strains are of great health importance subgroup of pathogenic *E. coli* that causes intestinal infection and extraintestinal side effects such as hemolytic uremic syndrome and, hemorrhagic purpura in the human (15, 16). Serotype O157: H7 is one of the most important members of Shiga toxin-producing group, known to be a causative agent of foodborne infections, often transmitted to human via the animals' origin foods (6-8, 10, 11).

In the present study, STEC strains were isolated from foods, such as minced beef, raw milk, and chicken, and also, the fecal samples of the human with gastroenteritis. The isolates were studied for the existence of virulence genes and genetic similarity, as well as their antibiotic resistance. The results showed that stool and food samples were contaminated with STEC strains. Among the isolates, 5 were identified as *E. coli* O157: H7, of which 2 isolates were from diarrhea and 3 isolates were from food samples. Also, the results showed that the prevalence of *E. coli* strains that produce Stx, other than O157: H7 serotype, was higher in patients and food samples. Other studies in Iran on animal origin foods such as meat and milk have shown that the prevalence of non-O157: H7 serotypes are higher than O157: H7 serotype (27, 28).

*E. coli* strains producing Shiga toxin have been isolated from dairy and meat products around the world. The presence of STEC in slaughtered beef samples was 13.7%, in Turkey (2009), (29). In another study Soma Sekhar et al. (2017) in India reported an 8% contamination in chicken meat with STEC strains (30). Also, the other study in India (2018) showed the presence of STEC strains in milk at 8.8% (31). In Ireland, Prendergast et al. (2009), detected *E. coli* O157 in 7.2% of the samples, including ground meat, and cow carcasses (32). Zhang et al. (2015), in southern China, reported the contamination of various types of meat with STEC strains at 4.1% (33). The difference in the contamination rate of STEC strains depended on variation in sample type, the number of samples, the sampling season, and the detection

**Table 4.** Virulence genes of *E. coli* isolated from diarrheal and food samples

| Samples | *E. coli* O157 | *E. coli* O157: H7 | *E. coli* Non-O157 |
|---|---|---|---|
| Feces | 1 (33.3%): *stx1* | 1 (50%): *stx1, stx2, eaeA, hlyA* | 2 (33.3%): *stx1* |
| | 2 (66.7%): *hlyA* | 2 (100%): *stx1, stx2, hlyA* | 4 (66.7%): *stx2, hlyA* |
| Raw Milk | 1 (33.3%): *stx1* | 1 (100%): *stx1, stx2, eaeA, hlyA* | 1 (100%): *stx2* |
| Chicken | 1 (33.3%): *stx2* | - | 1 (100%): *stx2* |
| Minced Meat | 1 (33.3%): *eaeA* | 1 (50%): *stx1, eaeA* | 1 (50%): *stx1, stx2* |
| | - | 1 (50%): *stx1, stx2, eaeA, hlyA* | 1 (50%): *stx2, hlyA* |

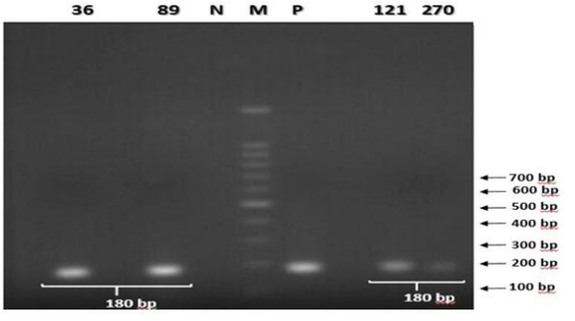


**Fig. 3.** PCR products for the *stx1* gene. M: 100bp DNA ladder,
N (Negative control), P (Positive control).

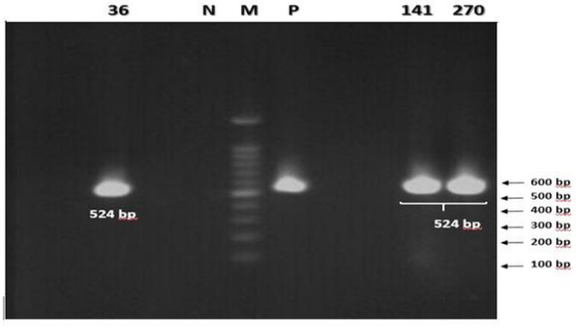


**Fig. 4.** PCR products for the *stx2* gene. M: 100bp DNA ladder,
N (Negative control), P (Positive control).

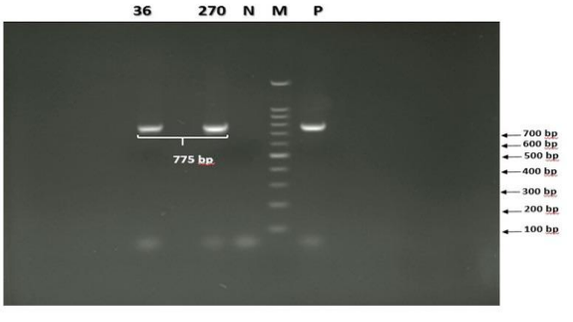


**Fig. 5.** PCR products for the *eaeA* gene. M: 100 bp DNA ladder,
N (Negative control), P (Positive control).

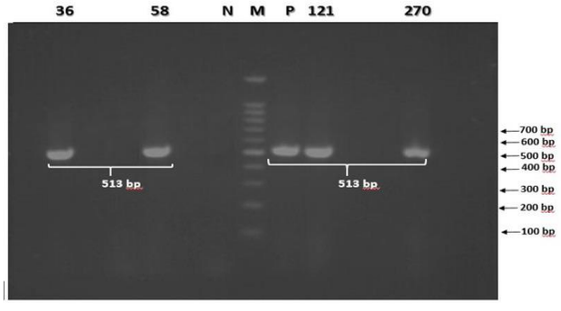


**Fig. 6.** PCR products for the *hlyA* gene. M: 100 bp DNA ladder,
N (Negative control), P (Positive control).

**Table 5.** Antibiotic resistance pattern in STEC strains isolated from diarrheal and food samples.

| Antibiotics | No (%) of resistance strain | |
|---|---|---|
| | Patients | Food samples |
| Ampicillin (AM/10 µg) | 6 (54.5%) | 9 (69.21%) |
| Trimethoprim-Sulfamethoxazole (SXT/25 µg) | 5 (45.5%) | 3 (23.07%) |
| Kanamycin (KAN/30 µg) | 1 (9%) | 2 (15.38%) |
| Gentamicin (GM/10 µg) | 0 (0%) | 0 (0%) |
| Chloramphenicol (CLR/30 µg) | 1 (9%) | 3 (23.07%) |
| Ciprofloxacin(CIPRS/5 µg) | 6 (54.5%) | 4 (30.76%) |
| Ceftazidim (CAZ/30 µg) | 5 (45.5%) | 8 (61.52%) |
| Nitrofurantoin (NIT/300 µg) | 0 (0%) | 3 (23.07%) |
| Nalidixic acid (NAL/30 µg) | 4 (36.5%) | 10 (76.9%) |
| Imipenem (IMP/10 µg) | 0 (0%) | (0%) |

method used.

The results of the current study defined that STEC strains isolated from patients and foods were resistant to ampicillin, nalidixic acid and ciprofloxacin. The resistance of the STEC to antibiotics in other countries, including South Korea (ampicillin and tetracycline), (34); Ireland (tetracycline, sulfonamides, and streptomycin), (32); Egypt (ampicillin, ciprofloxacin, gentamicin, and erythromycin), (35), are re-

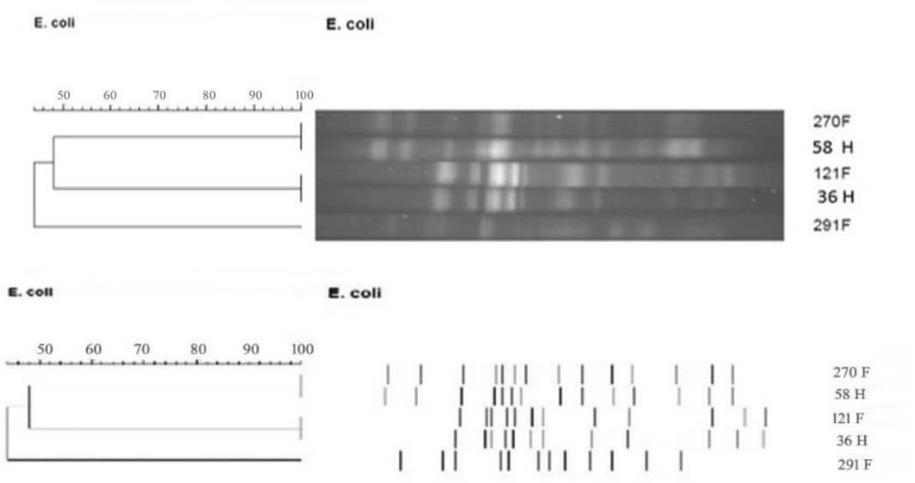


**Fig. 7.** PFGE patterns of *E. coli* O157: H7 strains isolated from foods and fecal samples

ported. Unlike other studies, no resistance to imipenem was identified in the present study. It seems that the high sensitivity to this antibiotic is due to the lack of access to this drug and its very limited use in the treatment of livestock and it is also more expensive than other antibiotics.

Genetic typing is a powerful tool for investigating outbreaks of infectious diseases by allowing the identification of sources of infection and routes of transmission of the organism. The molecular subtyping–based surveillance system for foodborne bacterial diseases using PFGE, has been used initiated by the CDC. Currently, PFGE is used for investigating outbreaks of disease in humans associated with *E. coli* O157:H7 (36, 37). However, PFGE subtyping data do not always provide valuable information to 'epidemiologists, even if multiple enzymes are used in the analysis (38).

The results of PFGE showed that there was 100% genetic similarity between strains isolated from diarrhea cases and foods. According to the results, similar patterns were found among human and meat isolates, cluster A, and also, the other human and milk isolates, cluster B. The strains in clusters A and B expressed the same virulence genes, while cluster C differed markedly in the expression of virulence genes.

Pulsed field gel electrophoresis typing results showed a close match between *E. coli* O157:H7 isolated from cattle products (meat and milk) and human samples in a similar pattern. In numerous studies, food of animal origin has been confirmed as an important route of disease transmission in outbreaks and sporadic infections of *E. coli* O157:H7 (39-42).

## CONCLUSION

According to the results of present study, STEC strains are the cause of 11% of infectious diarrhea cases that are resistant to certain antibiotics such as ciprofloxacin, ampicillin, ceftazidime and cotrimoxazole. In genetic comparison, there was a 100% similarity between *E. coli* O157: H7 isolated from the foods (beef and raw cow milk) and human gastrointestinal infections, which shows that in Iran, as the same as the other parts of the world, cow-origin foods are the most important sources of infection in humans.

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