International Journal of Enteric Pathogens

Int J Enteric Pathog. 2020 May;8(2):55-59 Original Article

http://enterpathog.abzums.ac.ir doi 10.34172/ijep.2020.12

Evaluation of *stx1, stx2, hlyA*, and *eaeA* Virulence Genes in *Escherichia coli* O157:H7 Isolated from Meat (Beef and Mutton) in Hamedan, Iran, During 2015-2016

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Published Online May 28, 2020

Keywords: *E. coli* O157:H7, Meat, PCR, Virulence factors

Abstract

Background and Objectives: Consuming raw or undercooked cattle meat is the most common transmission way of infection with *Escherichia coli* O157:H7. The present study aimed to identify virulence genes *stx*1, *stx*2, *hlyA,* and *eaeA* in *E. coli* isolated from meat samples (beef and mutton) in Hamedan during 2015 and 2016.

Materials and Methods: For this purpose, the swabs were randomly taken from 160 meat samples including 80 beef samples and 80 mutton samples from butcher shops. Isolation and identification of *E. coli* cells were conducted by culturing the swab samples on MacConkey agar and Eosin methylene blue agar media. Then, the identity of the suspected *E. coli* O157:H7 colonies was investigated by a multiplex PCR assay and eventually, the isolates were evaluated for the presence of *stx1*, *stx2*, *hlyA*, *eaeA* virulence genes.

Results: The results showed that out of 160 cultured samples on the selective media, 60 samples (37.5%) were contaminated with *E. coli*. O157:H7, O157, and H7 strains were identified using PCR, among which only *E. coli* O157:H7 possessed all four virulence factor encoding genes.

Conclusion: The results of this study showed that beef could be a reservoir for *E. coli* O157:H7, and it may be involved in the transmission of this pathogen to humans.

Received January 10, 2020; **Revised** May 13, 2020; **Accepted** May 20, 2020

Background

Escherichia coli (*E. coli*) is a part of the normal microflora of the digestive tract of mammals, certain strains of which are associated with gastrointestinal diseases in both humans and warm-blooded animals[1]. The most important *E. coli* serotype is *E. coli* O157:H7, which has drawn widespread attention due to its toxin-producing capability[2]. Production of Shiga toxin by some of the *E. coli* strains, known as Shiga-toxin producing *Escherichia coli* (STEC), has been reported in several outbreaks, including mild diarrhea, hemorrhagic colitis (HC), and hemolytic uremic syndrome (HUS)[3].

The pathogenicity of *E. coli* O157 is related to a variety of virulence factors, including Shiga toxins with two major groups, stx1 and stx2[4], Intimin (encoded by the *eae* gene), which is involved in the attachment of *E. coli* to the enterocyte and caused attaching and effacing (A/E) lesions in the intestinal mucosa[5], and *HlyA* (plasmid gene EHEC-*hlyA*), which is an exotoxin that lyses erythrocytes and different cells, promoting iron acquisition for microorganism nutrition[6].

Epidemiologic investigations demonstrated that O157 is the primary causative agent for HC and HUS in humans. However, 6 non-O157 O groups (O26, O45, O103, O111, O121, and O145) have been reported in human clinical cases[7].

Domestic ruminants, particularly cattle, sheep, and goats, are considered the principal reservoir of STEC strains. Nevertheless, other domestic animals, including pigs, poultry, cats, and dogs, may also act as reservoirs for this microorganism[8]. *E. coli* O157 outbreaks are related to the consumption of raw or undercooked meat, meat products, unpasteurized dairy products, and vegetables or water contaminated with animal feces[9]. Additionally, infection with *E. coli* O157 can be acquired through contaminated drinking or swimming water, close contact with infected animals, and person-to-person transmission[10].

Since the incidence of gastroenteritis due to STEC is increasing and considering its important hygienic, social, and cultural aspects, there is an increasing demand for improved diagnostic procedures for the detection of

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STEC in fecal samples and, particularly, in foods such as meat and dairy products[11,12]. Nowadays, polymerase chain reaction (PCR) has become a useful fast, specific, sensitive, and comparatively inexpensive diagnostic tool for the detection of *STX* encoding genes in different samples containing even a small amount of STEC[9].

To our knowledge, this is the first study that aimed to identify virulence genes *stx*1, *stx*2, *hlyA,* and *eaeA* in *E. coli* O157:H7 isolated from red meat (beef and mutton) in Hamedan. Because meat and food products that are contaminated with animal feces are probably the primary sources of the *E. coli* O157: H7 infection, the present study focused on the detection of *E. coli* O157:H7 and its virulence genes in meat samples by PCR assay.

Materials and Methods

Sample Collection

This cross-sectional descriptive study was conducted during 2015 and 2016. The sampling was done according to the simple random sampling method, and based on previous prevalence rates reported, the sample size was calculated to be 160 cases[13,14]. A total of 160 swab samples (beef n=80 and mutton n=80) were randomly collected from butcher shops in Hamedan. Sampling was done according to the instruction of the Institute of Standards and Industrial Research of Iran, using swabs moistened with 0.1% peptone water. The samples were immediately transported to the Faculty of Veterinary Medicine at Bu Ali Sina University in ice-cooled containers.

Bacterial Isolates

The swabs collected from red meat samples (beef and mutton) were immediately inoculated on MacConkey agar (Merck, Germany) to evaluate the ability of lactose fermentation. Because the bacterium is able to ferment lactose, lactose-fermenting colonies were presumptively considered as *E. coli*[13]. Then, these grown reddish-purple colonies were transferred to Eosin Methylene Blue agar (EMB agar; Merck, Germany) for final confirmation and incubated at 37°C overnight (dark purple colony with a green metallic sheen)[15].

Multiplex PCR for Detection of stx1, stx2, hlyA, eaeA Genes

DNA Extraction

To investigate O157 and H7 encoding genes as well as genes responsible for virulence factors including *stx1*, *stx2*, *hlyA*, and *eaeA*, PCR technique was used. First, DNA was extracted from suspected isolates using the boiling method. Briefly, about 5 mL of an overnight TSB culture of each *E. coli* isolate was precipitated and resolved in 200 µL of sterile distilled water and vortexed. After incubating at 100°C in a water bath for 10 minutes, the solution was centrifuged at 10000×g for 2 minutes. Then, supernatants were collected as the source of extracted DNA samples[11].

Primer Sequences

PCR was performed using previously described primers, as presented in Table 1[16].

DNA Amplification

The PCR reactions contained 12.5 µL of a commercial PCR MasterMix (Ampliqon, Denmark), 0.25 µL of each primer with a concentration of 0.5 µM (four primer pairs), and 7 µL of the extracted DNA as the template in a final volume of 25µL. The condition of cycles in a thermocycler (Astec, Germany) was as follows: the samples were subjected to 35 PCR cycles, each cycle including denaturation at 95°C for 1 minute, annealing at 65°C for 2 minutes for the first 10 cycles, decrementing to 60°C by cycle 15, and elongation at 72°C for 1.5 minutes. The final extension was done at 72°C for 2.5 minutes from cycles 25 to 35. After electrophoresis of PCR products on 2% agarose gel and ethidium bromide staining, amplified products were visualized by UV illumination. *E. coli* O157:H7 (ATCC-35150; prepared from the bacteria collection of the Faculty of Veterinary Medicine of Tehran) was used as positive control and a sample without any DNA as a negative control[11,16].

Statistical Analysis

The findings were analyzed by Pearson's chi-square test using SPSS (version 16.1). Differences in the isolation rate were considered statistically significant at $P < 0.05$.

Results

Out of the 160 collected samples, 60 samples (37.5%) on MacConkey medium were lactose-fermenting isolates. Among them, 15 samples (25%) belonged to beef, and 45 samples (75%) belonged to mutton. Lactose-fermenting colonies on EMB agar strongly fermented lactose and showed a green metallic sheen on this medium. These

Table 1. The Primer Sequences Used for PCR Reactions[16].

Primer	Oligonucleotide sequence (5′- 3′)	Size of product (bp)
*stx1*F	ATAAATCGCCATTCGTTGACTAC	180
*stx1*R	AGAACGCCCACTGAGATCATC	
*stx2*F	GGCACTGTCTGAAACTGCTCC	255
*stx2*R	TCGCCAGTTATCTGACATTCTG	
*eaeA*F	GACCCGGCACAAGCATAAGC	384
*eaeA*R	CCACCTGCAGCAACAAGAGG	
*hlyA*F	GCATCATCAAGCGTACGTTCC	534
*hlyA*R	AATGAGCCAAGCTGGTTAAGCT	
*O157*F	CGGACATCCATGTGATATGG	259
*O157*R	TTGCCTATGTACAGCTAATCC	
*H7*F	GCGTGTCGAGTTCTATCGAGC	625
*H7*R	CAACGGTGACTTTATCGCCATTCC	

isolates were selected for final confirmation by PCR. In the initial evaluation by the PCR test, out of the 60 positive isolates in culture examination, one isolate (1.67%) had both O157 and H7 encoding genes simultaneously and was characterized as the *E. coli* O157: H7 strain, while only one isolate (1.67%) had the O157 encoding gene and was considered as *E. coli* O157: non motile (NM). In addition, one isolate (1.67%) possessed the H7 encoding gene (Figure 1).

A Multiplex PCR assay was used to identify virulence factors (*stx1, stx2, hlyA, eaeA*). Of the three isolates (O157:H7, O157, H7) only O157: H7 had all four virulence factor encoding genes, and the other two isolates did not form DNA bands for any of the four genes (Figure 2).

Finally, out of the 57 non-O157:H7 strains , 18 isolates (31.5%) had at least one *stx1*, *hlyA*, and *eaeA* genes. None of the non-O157:H7 strains possessed *stx2* gene (Table 2).

Discussion

E. coli serotype O157:H7 is a severe threat to public health and impose a considerable economic burden on the food industry[17]. Since cattle are the main reservoirs of STEC, beef and beef products are reported to be the most common food vehicles attributed to human disease [18]. Many studies determined the prevalence of *E. coli* O157:H7 in cattle carcasses, which ranged from 0.0% to 27.8% (up to 68% in heifers)[19]. Many protocols for isolation of the *E. coli* O157:H7 from food samples have been proposed. To date, different PCR assays have been described for detecting the main virulence factor genes such as *stx1*, *stx2*, *hlyA*, and *eaeA*[13]. Osek (2003) performed multiplex PCR assay on 202 *E. coli* isolates form cattle and children at the National Institute for Veterinary Research of Poland and found that 25 STEC isolates (12.4%) were *stx* positive and 20 STEC isolates had *eaeA* and *ehlyA* virulence genes[20].

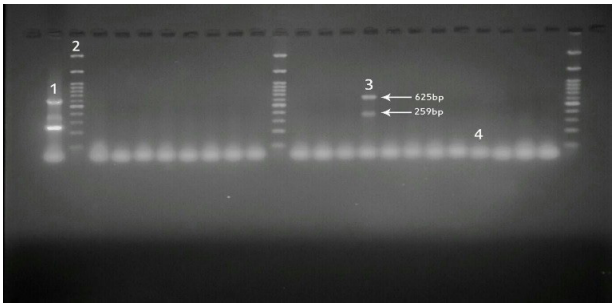

Figure 1. Electrophoresis of PCR Products for Detection of O157:H7, H7 Strains. Lane 1: Positive Control O157: H7 Strain Containing all Four Genes (*stx1, stx2, hlyA, eaeA*), Lane 2: a 100-bp DNA Ladder, Lane 3: Positive Isolates for O157:H7, H7, Lane 4: Negative Control.

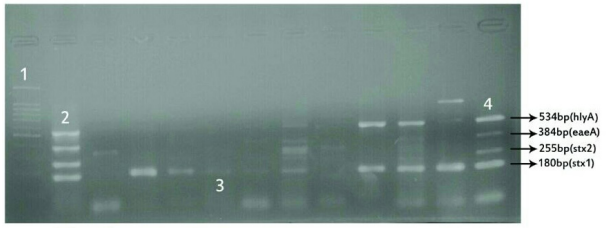

Figure 2. Detection of *stx1, stx2, hlyA,* and *eaeA* Genes in O157: H7 Isolate by Multiplex PCR Assay. Lane 1: a 100-bp Ladder, Lane 2: Positive Control, Lane 3: Negative Control, Lane 4: *E. coli* O157: H7 Isolate Contained All Four Virulence Genes.

Table 2. Result of PCR Assay for stx1, hlyA, and eaeA Genes in Non-O157:H7 Isolates

Virulence profile	Number of isolates (%)
stx1	5 (27.8%)
stx1+hlyA	9 (50%)
stx1+eaeA	2 (11.1%)
eaeA	2 (11.1%)
Total	18 (100%)

In the present study, a multiplex PCR was used to identify virulence factors (*stx1*, *stx2*, *hlyA*, and *eaeA*). Out of 160 *E. coli* isolates tested, 18 non-O157: H7 strains had at least one virulence factor-encoding gene, and only one O157: H7 isolate was identified to have all four virulence factor encoding genes *stx1*, *stx2*, *hlyA*, and *eaeA* (Table 2). The contamination rate of beef with *E. coli* O157:H7 was 1.67% (one of the 60 *E. coli* isolates) in Hamedan. In a similar study, Jamshidi et al reported that only one of 100 ground beef samples was contaminated with *E. coli* O157:H7 using PCR assay in Mashhad[13]. However, as reported by Rahimi et al, the contamination rate of slaughtered cattle in Isfahan was 4.6%, indicating a higher rate compared to the present study[21]. Moreover, Shekarfroush et al in Shiraz and Jafaryan-Sedigh et al in Esfahan showed that the contamination rate of mutton with *E. coli* O157:H7 was 3.92 and 6.8%, respectively[22,23]. In another research carried out by Hajian et al, the contamination rate of beef was found to be 2.2%[24], which was higher than that obtained in the present study. The contamination rate of beef reported in the present study in Hamedan (1.67%) was higher than that in Ireland (0.0%) and lower than that in the Netherlands (10.4%), England (13.4%), and the USA (28%)[21]. Differences in experiment results may be due to the sampling method (samples of carcass meat, meat products packed in supply centers), size and number of samples, and isolation method performed in the present study[17]. The current study is the first report on the contamination of red meat in Hamedan with *E. coli* O157:H7, suggesting that consuming undercooked meats can contribute to the transmission of this pathogen to humans. Therefore, control, precise monitoring, and observance of hygienic principles during slaughter, transportation, and storage can be effective measures in reducing cross-contamination. In addition,

due to the risks of the presence of this bacterium in food, it is necessary to study the etiologic characteristics of this bacterium in foodstuffs and ways of its transmission to humans. Additionally, it is recommended that more comprehensive studies should be done in other regions of the country[10].

Conclusion

This study showed that red meat can be a reservoir for *E. coli* O157: H7 and it may act as a vehicle for transmission of this pathogen to humans. On the basis of the data presented here, it is necessary for the consumer to be aware of the potential health risks related to the consumption of meats that are not heated or partially heated before consumption.

Authors' Contributions

FB performed laboratory experiments. MPA presented the original idea, performed laboratory experiments, and wrote the manuscript. PM provided technical support and wrote the paper. AA wrote the paper.

Ethical Approval

Not applicable.

Conflict of Interest Disclosures

Authors declare no conflict of interests.

Financial Support

Bu-Ali Sina University, Hamedan, Iran.

Acknowledgments

The authors are grateful to Bu-Ali Sina University (Hamedan, Iran) for financial support.

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