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Isolation and Molecular Characterization of Non-Sorbitol Fermenting *Escherichia coli* Isolated From Fresh Ground Beef

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Abstract

Background: *Escherichia coli* O157:H7 (EHEC) has been traditionally associated with foodborne infections from consumption of foods with animal origin such as ground beef and burgers.

Objectives: The objective of this study was to investigate the contamination of fresh ground beef with non-sorbitol fermenting *Escherichia coli* and presence of virulence genes in isolates obtained from butchers located in Ahvaz, Iran.

Materials and Methods: A total of 200 fresh ground beef were sampled during a six-month period. All samples were enriched in Tryptic Soy Broth (TSB) with novobiocin and plating on Cefixime Telluride-Sorbitol MacConkey (CT-SMAC). The suspected colonies were subjected to Polymerase Chain Reaction (PCR) analysis to identify virulence genes containing rfbE O157, flic H7, stx1 and stx2 genes.

Results: Overall, 1.5% of ground beef samples were contaminated with the O157 *E. coli* strain meanwhile 1% of samples contained the O157:H7 strain and 0.5% of samples had the O157:H7 with virulent stx1 and stx2 genes.

Conclusions: The observed results indicated the necessity of good care in abattoir, butcheries and during food distribution, mainly ground beef. Also, a zero tolerance policy could be useful to control *E. coli* O157 in meat products nationwide.

Keywords: Polymerase Chain Reaction (PCR), Isolation, Meat Products, Molecular Characterization, *Escherichia coli*

1. Background

Enterohemorrhagic *Escherichia coli* O157:H7 (EHEC) is one of the most important foodborne pathogens that produces verotoxin and has been traditionally associated with foodborne infection from consumption of foods with animal origin, particularly those originating from cattle, such as ground beef and burgers (1). Hemorrhagic Colitis (HC) and Hemolytic Uremic Syndrome (HUS) are two chronic and potentially fatal illnesses caused by EHEC (2). Consumption of undercooked minced beef is the most common route of transmission of EHEC. Thousands of illnesses and hospitalizations and hundreds of deaths due to this bacterium has been reported (3).

To the best of our knowledge, isolation of the pathogenic organism from ground beef and meat products in various countries such as Republic of Ireland (4), Egypt (5), Switzerland (6), Turkey (7), Mexico (8), USA (9), Brazil (10) and China (11) has been documented. In Iran, there has been a number of assays on the isolation and the prevalence of *E. coli* O157:H7 in ground beef and beef products. Jafareyan reported that 10 (6.8%) of the 148 ground beef samples were contaminated with *E. coli* O157:H (12). Shekarforoush et al. detected verotoxigenic *E. coli* O157:H7 in six (3.92%) of the 153 sheep carcass samples (13) and Shahrokhabadi found that six (4.05%) of the 148 bovine carcasses samples were contaminated With the bacterium (14).

The pathogenicity of *E. coli* O157:H7 is affected by several virulence factors. The main factor contributing to the pathogenicity is its ability to produce potential cytotoxins called Shiga-toxins (Stx), encoded by stx1 and stx2 genes (15). Other described virulence factors include intimin, encoded by the eaeA gene and EHEC hemolysin encoded by the EHEC hlyA gene (16). Also, it has been reported that non-O157 strains could be verocytotoxigenic (17, 18).

2. Objectives

The aim of the present study was to determine the prevalence of viable Non-Sorbitol Fermenting (NSF) *E. coli* containing rfbE O157 and flic H7 genes and, to detect the presence of the stx1 and stx2 genes in isolates from ground beef samples obtained from butcheries in Ahvaz city, Iran.

3. Materials and Methods

3.1. Sample Preparation

A total of 200 samples of fresh ground beef were ob-

tained from butcheries located in different parts of Ahvaz during a six-month period. Samples were transported to the laboratory under cold conditions. Samples were analyzed according to *E. coli* O157:H7 isolation procedures on the same day.

3.2. Isolation and Identification of Non-Sorbitol Fermenting Escherichia coli

All samples were examined for the presence of NSF *E. coli* by regular procedures (19). Ten grams of each sample was added to 90 mL Tryptone Soy Broth (TSB) (Quelab, Canada) supplemented with novobiocin (20 mg/L, Sigma) and homogenized. After incubation for 24 hours at 37˚C, a 100-µL suspension was spread onto Cefixime Telluride-Sorbitol MacConkey (CT-SMAC) agar (scharlau, Spain) supplemented with cefixime (0.25 mg/mL) and tellurite potassium (2.5 mg/mL). Plates were incubated for 24 hours at 37˚C and examined for typical *E. coli* colonies (colorless, circular with brown center). The suspected NSM *E. coli* colonies were plated on both Eosin-Methylene Blue agar (EMB) (Scharlau, Spain) and Tryptone Bile x-Glucoronide (TBX) (Merck, Germany) and were incubated at 37°C for 24 hours. Also, the suspected isolates were subjected to Polymerase Chain Reaction (PCR) analysis to identify virulent genes.

3.3. The Polymerase Chain Reaction Procedures

3.3.1. DNA Extraction

Extraction was performed using a modification of the method previously described by Lopez-Saucedo et al. (20). Presumptive *E. coli* colonies were separately grown overnight in 5 mL of TSB at 37°C. These cultures were centrifuged (Hitachi 1110, Germany), the pellet was resuspended in 1 mL of sterile distilled water, and samples were heated at 100°C for ten minutes. After heating, the suspension was again centrifuged and the supernatant was used as the PCR template.

3.3.2. Polymerase Chain Reaction and Electrophoresis

Presumptive colonies in two steps were subjected to the PCR assay for amplification of four pairs of specific primers, including: (FliC) H7 and O157 in first and stx1 and stx2 in the second steps, according to the following program for both steps: initial denaturation at 94˚C for three minutes, and then 35 cycles comprised of denaturation at 94˚C for 45 seconds, annealing at 60˚C for 45 seconds, and an extension at 72˚C for 60 seconds. Following this, a final extension at 72˚C for five minutes was carried out. Each PCR tube contained 25 µL of reaction mixture, consisting of 2.5 µL of PCR buffer (10x), 1.5 µL $MgCl_2$ (50 mM), 1 µl dNTP (10 mM), 0.5 µl Taq polymerase (2.5 U), 4 µL of a mixture of the two forward and revised primers (15 µM), 10.5 µL of ddH_2O and 5 µL of template extracted DNA. The mixture was then processed in a thermocycler (Bioer Technology Co., China). The targets, primer sequences and amplicon sizes for the PCR products are shown in Table 1.

The amplified PCR products were detected by electrophoresis (Paya pajoohesh, Iran) and staining, and visualized under UV light illumination (UVT-20 SL, Iran).

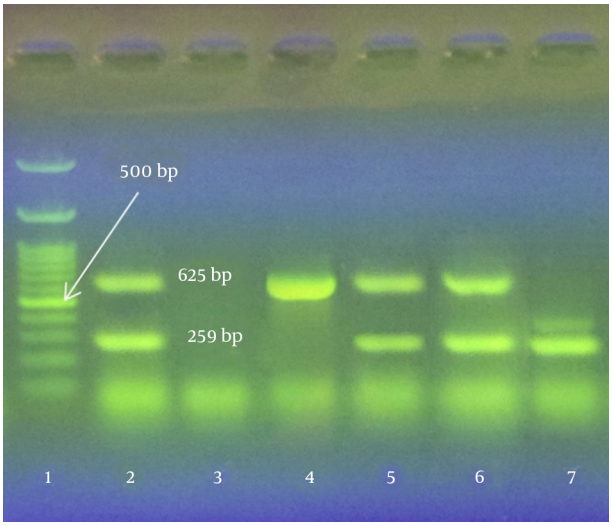
4. Results

The present study focused on identification of virulent genes in NSF isolates from ground beef in Ahvaz city. A total of 23 strains of NSF *E. coli* were recovered from 200 meat samples (11.5%) by CT-SMAC culture agar and plating on EMB agar. Among these, 14 strains were colorless on TBX medium and probably had negative ß-glucuronidase enzyme activity. Our findings showed that two strains contained both O157 and H7 genes, one strain was O157 and nine strains were only H7 positive. Virulent stx1 and stx2 genes were found only in an O157:H7 strain (Figures 1 and 2).

Table 1. List of Target Genes, Sequence of Primers and Product Size (bp)

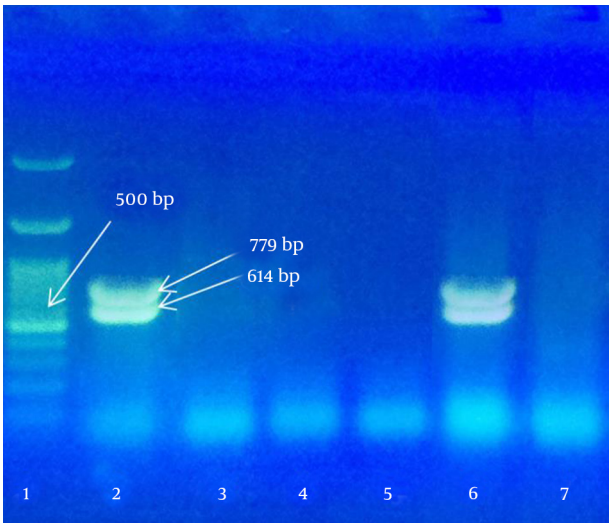
Primer	Size (bp)	Sequence	Reference
O157	259		(21)
		F: 5 –CGGACATCCATGTGATATGG-3	
		R: 5-TTGCCTATGTACAGCTAATCC -3	
(FliC) H7	625		(22)
		F : 5- GCGCTGTCGAGTTCTATCGAGC -3	
		R: 5- CAACGGTGACTTTATCGCCATTCC -3	
STX1	614		(23)
		F: 5 – ACACTGGATGATCTCAGTGG-3	
		R: 5- CTGAATCCCCCTCCATTATG -3	
STX2	779		(23)
		F : 5- CCATGACAACGGACAGCAGTT -3	
		R: 5- CCTGTCAACTGAGCAGCACTTTG -3	

Figure 1. Polymerase Chain Reaction Results on Gel Electrophoresis



1, Ladder 100 bp plus; 2, positive control (*E. coli* O157:H7 strain ATCC 43895); 3, negative control; 4, H7 positive isolate; 5, O157:H7 isolate; 6, O157:H7 isolate; 7, O157 isolate.

Figure 2. Polymerase Chain Reaction Results for stx Detection on Gel Electrophoresis



1, Ladder 100 bp plus; 2, positive control (*E. coli* O157:H7 strain containing stx1 and stx2 genes; culture collection of Dep. of pathobiology, University of Shiraz); 3, negative control; 4- H7 isolate; 5, O157:H7 isolate; 6, O157:H7 isolate containing stx1 and stx2 genes; 7, O157 isolate.

In conclusion, 1.5% of ground beef samples contaminated the O157 *E. coli* strain meanwhile 1% of samples were O157:H7 positive and 0.5% of samples were O157:H7 positive containing virulent stx1 and stx2 genes.

5. Discussion

Beef is one of the favorable consumed meats in the world. It has been noted that beef carcass and ground beef are the most important sources of *E. coli* O157:H7 (24). Infections caused by these bacteria could lead to diarrhea, hemorrhagic colitis or Hemolytic Uremic Syndrome (HUS) (25). The bacterium recognized as low dose foodborne pathogens (17).

In our study, serovar O157:H7, not motile O157 and non-O157 *E. coli* were isolated from two (1%), one (0.5%) and 20 (10%) ground beef samples, respectively, which indicates the risk of consumption of this kind of food if severely undercook. Several studies have shown that *E. coli* O157:H7 and other Shiga-Toxin *E. coli* (STEC) are present in meat products; mostly beef products. For example Cagney et al. (4) investigated the prevalence and numbers of *E. coli* O157:H7 in minced beef and beef burgers in supermarkets and butcheries in the Republic of Ireland. Overall, *E. coli* O157:H7 was recovered from 43 samples (2.80%). In France, Vernozy-Rozand et al. reported that 0.12% (4/3450) of samples were positive for *E. coli* O157:H7 in large-scale processed minced beef (26). *Escherichia coli* non-O157, *E. coli* O157: NM (not motile) and *E. coli* O157:H7 were isolated from 53 (20.5%), 13 (5%) and seven (2.7%) of the 258 beef carcasses, respectively, sampled by Varela-Hernandez in Mexico (8). Ahmed and Shimamoto recovered *E. coli* O157:H7 from 4.3% ground beef collected from butcheries in Egypt (5). Fantelli and Stephan detected O157:H7 in 2.3% of 213 ground beef samples (6), while this pathogen was isolated from 7.6% of 251 ground beef samples by Sarimehmetoglu, in Turkey (7). In Argentina, Chinen isolated *E. coli* O157:H7 from 3.8% of 161 ground beef samples (27). Other studies found very different results, ranging from 16.8% (50/296) *E. coli* O157:H7 samples in Washington State, USA (28) to 0%, as determined by the study of Tarr et al., which did not recover the pathogen from 1400 retail ground minced beef samples from six stores in Seattle, USA (29).

A few studies have indicated that the isolation of the bacterium in meat products in Iran. For example Rahimi (30) reported a high prevalence of *E. coli* O157:H7 in beef samples (8.2%), followed by water buffalo (5.3%), sheep (4.8%), camel (2.0%) and goat (1.7%). Again, in another study from Iran, high incidence of *E. coli* O157:H7 in ruminant's meat samples was reported by Momtaz, where, 238 (29.02%) samples were positive for the presence of *E. coli*. All of the isolates had more than one virulence gene including stx1, stx2, eaeA and hly (31). In South-West of Iran, *E. coli* O157:H7 was found in six (3.92%) of 153 sheep carcasses (13). In Isfahan, Jafareyan-Sedigh reported that 10 (6.8%) of 148 sheep meat samples were contaminated with *E. coli* O157:H7 (12). Shahrokhabadi recovered the pathogen from six (4.05%) of 148 cattle carcasses in Slaughterhouse of Rafsanjan (14).

Various factors such as verocytotoxin (encoded by stx1 and stx2), a protein called intimin (encoded by eaeA gene) and enterohemolysin (encoded by EHEC hlyA gene) are linked to the pathogenesis of *E. coli* O175:H7 (16, 21, 31). In the current study, 20 non-O175 *E. coli* with no virulent stx1 and stx2 genes were isolated. Meanwhile, it has been reported that non-O157 strains could also be verocytotoxi-

genic (17, 18). Our data showed that 14 strains were colorless on TBX medium and were assumed to be the O157 strain yet only three of them contained O157 genes. It was concluded that this medium could not be a reliable medium for the detection of O157 strains. Also, the H7 gene was identified in nine non-O157 strains and may belong to the O55 strain because the flic genes of O55:H7 and O157:H7 strains are closely related (32).

Generally, 1.5% of ground beef samples were contaminated with O157 *E.coli*. These kinds of products may pose risks to the health of consumers if eaten raw or undercooked. To reduce risk, the incorporation of all agents involved in the beef supply chain is necessary. Also, application of Hazard Analysis of Critical Control Points (HACCP) from the farm to the abattoir should be applied by governmental authorities. Consumers and retailers should be educated about the disease and methods of prevention. A zero tolerance policy could be useful to control *E. coli* O157 in meat products in the country.

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Footnotes

Authors' Contribution: Siavash Maktabi: study design, management, supervision and writing; Mehdi Zarei: advising; Hooriyeh Mohammadpour: sampling, processing and performing the conventional and molecular procedures.

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