

Antibiotic resistance of Verotoxigenic *Escherichia coli* isolated from vegetables

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Abstract

Introduction: Human gastrointestinal disease caused by verotoxigenic *Escherichia coli* has been diagnosed for recent decades. *Escherichia coli* O157:H7 is the most important serotype of verotoxigenic *Escherichia coli* that cause hemolytic uremic syndrome and hemorrhagic colitis in humans. This study was conducted to determine the occurrence of verotoxigenic *E. coli* and antibiotic resistance of the isolates from vegetables.

Materials and methods: A total of 500 fresh vegetable samples were collected randomly from retail shops in Shahrekord, Iran. *E. coli* was isolated and identified using bacteriological and biochemical tests. PCR method was used to identify the *rbfE*, *stx1*, *stx2* and *eae* genes. Also, antibiotic resistance of the isolates was determined by disk diffusion method.

Results: The results represented that among 25 isolates possess virulence genes, 40, 12 and 4% of the isolates contained *eaeA*, *STx2*, and both genes, respectively. But none of them contained *H7*, *STx1*, and *rbfE* genes. The antibiotic resistance pattern demonstrated that the isolates were highly resistant to Gentamycin and cefotaxime.

Discussion and conclusion: The results of this study showed that the presence of verotoxigenic *E.coli* in vegetables; and high resistance of the isolates to antibiotics could be hazardous for public health.

Key words: Verotoxigenic *E.coli*, Virulence genes, Antibiotic resistance, Vegetables

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Introduction

Shiga toxin-producing *Escherichia coli* (STEC) is currently considered as an important food-borne pathogen. The serotype O157: H7 is a widespread Enterohemorrhagic *Escherichia coli* (EHEC) serotype that causes several diseases such as hemorrhagic colitis, hemolytic uremic syndrome and thrombocytopenic purpura. The pathogenic capacity of STEC is due to a number of virulence factors such as Shiga toxins (*Stx1* and *Stx2*) and intimin (1). In fact, the ability of EHEC to colonize in human and animal intestinal mucosa and cause disease is associated with a number of virulence factors, including expression of Shiga toxins (Stx) (2) and the capacity to induce attaching/effacing (A/E) lesions (3). A/E lesions are characterized by intimate bacterial attachment to the host cell membrane and destruction of microvilli at the site of bacterial adhesion. Fresh vegetables can become contaminated in different points in the food processing chain. Thus, knowledge of survival and persistence of enteric pathogens on edible crops and during production provides information for agricultural practices. Leaves are inhabited by diverse highly-adapted microbes and are colonized frequently by transient microorganisms, including human pathogens (4).

A wide range of animal species are known to carry STEC and EHEC strains, but ruminants are the most important natural reservoir and excrete these bacteria through their feces (5). The main routes of infection transmission are person-to-person

and consumption of contaminated meat and milk. Moreover, ingestion of contaminated vegetables or water and direct contact with animals or soil are associated with EHEC-associated outbreaks. It has been reported that human infections can result from ingestion of fewer than 100 viable EHEC cells (5). Moreover, EHEC and STEC can persist and remain infectious for several weeks in slurries, farmyard manure and sewage sludge as well as on pasture land (6- 8).

The aim of this study was to identify the virulence genes and antibiotic resistance of the verotoxigenic *E. coli* isolated from vegetables.

Materials and methods

Sample collection: A total of 500 fresh vegetable samples including *Mentha*, *Ocimumbasilicum*, *Lepidium sativum*, *Allium ampeloprasumpersicum*, and *Allium fistulosum* were collected randomly from retail shops. **Detection of *E. coli*:** A portion of vegetable sample (100 g) was washed with 200 ml of sterile distilled water. 100 ml of raising water was centrifuged, 1ml of sediment transferred to EC broth medium and incubated at 37°C for 24 h. Then, 0.1 ml of EC broth was transferred to MC agar and incubated at 37°C for 24 h. Suspected colonies were selected and differential biochemical tests such as IMViC and culture on Eosin methylen blue medium were conducted to confirm *E.coli*. (9).

Identification of virulence genes by PCR: In order to identify the virulence genes, PCR assay was performed. *E. coli* colonies were subcultured in trypton soy broth. The

bacterial cells were suspended in 250 µl of sterile water and boiled at 100°C for 5 min to release the DNA, and the aliquot was centrifuged in 1200 rpm for 3 min. The supernatant was used in the PCR as template (10).

The strain of *E.coli* O157:H7 was prepared from microbiology laboratory of veterinary medicine of Tehran University as a positive control and sterile distilled water was used as a negative control. Sequences and predicted sizes of amplified products for the specific oligonucleotide primers are shown in Table 1. PCR was used for detection of *stx1*, *stx2*, *eae A*, *H7* and *rfbE* genes. Amplification of bacterial DNA was performed using 30µl volumes containing 50 ng of the prepared sample supernatant, 150 ng of the oligonucleotide primers, 0.2 mM (each) dATP, dGTP, dCTP, dTTP, 10 mM Tris-HCl (pH 8.8), 1.5 mM MgCl₂, 50 mM KCl and 1 U of Taq DNA polymerase (Bioline, London, United Kingdom).

The conditions performed in a thermal cycler (Hybaid, Southampton, United Kingdom) were 94°C for 2 min followed by 35 cycles of 94°C for 1 min, 55–64°C for 1 min and 72°C for 1 min. The

amplified products were visualized by standard submarine gel electrophoresis using 10 µl of the final PCR product on a 2% agarose gel in 0.5% TBE buffer. The samples were electrophoresed for 40 min at 100 V. Amplified DNA fragments of specific sizes were located by UV fluorescence after staining with Ethidium bromide (10).

Antibiogram tests: The antibiotic susceptibility of the isolates contained virulence genes were performed by disc diffusion method. The isolates were inoculated in TSB medium and incubated for 24 h at 37°C. After observing turbidity in TSB medium, a swab of bacteria was plated onto Mueller Hinton agar medium and antibiotic disks containing Ampicillin 10, Ciprofloxacin 5, Tetracycline 30, Trimethoprim-sulfamethoxazol 20, Cefotaxime 30 and Gentamicin 10, (Padtan Teb, Co) were placed in the plates. Plates were incubated for 24h at 37°C. The zone of growth inhibition for each antibiotic disc was measured. The zone diameters around all disks were interpreted using the recommendation of the Clinical and Laboratory Standards Institute (CLSI).

Table 1- Characterization of primers for detection of virulence genes of isolated *E. coli* (Rey 2006), (21).

| Gene target | Primers | Oligonucleotide Sequence (5'-3') | Fragment (bp) | Annealing temperature |
|-------------|------------------|---|---------------|-----------------------|
| <i>rfbE</i> | O157-R O157-F | 5'-CGTGGTATAGCTACTGTACC-3' 5'-CGCTGAATGTCATTCGCTCTGC-3' | 259 | 58 |
| <i>STx1</i> | VT1-R VT1-F | 5'-CGTGGTATAGCTACTGTAC<C>-3' 5'-CGCTGAATGTCATTCGCTCTG<C>-3' | 302 | 58 |
| <i>STx2</i> | VT2-R VT2-F | 5'-CTGCTGTGACAGTGACAAAACG<C>-3' 5'-CTTCGGTATCCTATTCCCG<G>-3' | 516 | 64 |
| <i>eaeA</i> | EAE-R EAE-F | 5'-GCGGTATCTTCGCGTAATCGC<C>-3' 5'-GAGAATGAAATAGAAGTCG<T>-3' | 775 | 50 |
| <i>H7</i> | H7-R H7-F | 5'-CAACGGTGACTTTATCGCCATTCC-3' 5'-GCGCTGTCGAGTTCTATCGAGC-3' | 625 | 60 |

Table 2. The *E. coli* isolates possess virulence genes from vegetables.

| Type of sample | No of Sample | <i>E. coli</i> | <i>rfbE</i> | <i>H7</i> | <i>STx2</i> | <i>STx1</i> | <i>eaeA</i> | <i>eaeA and STx2</i> | Total genes |
|-------------------------------------|--------------|----------------|-------------|-----------|-------------|-------------|-------------|----------------------|-------------|
| <i>Ocimum basilicum</i> | 100 | 6 | - | - | - | - | 3 | 1 | 4 |
| <i>Mentha piperita</i> | 100 | 7 | - | - | 1 | - | 3 | - | 4 |
| <i>Lepidium sativum</i> | 100 | 4 | - | - | - | - | 2 | - | 2 |
| <i>Allium fistulosum</i> | 100 | 5 | - | - | - | - | 1 | - | 1 |
| <i>Allium ampeloprasum persicum</i> | 100 | 3 | - | - | 2 | - | 1 | - | 3 |
| Total | 500 | 25 (5%) | - | - | 3 (12%) | - | 10 (40%) | 1 (4%) | 14 (56%) |

Results

Overall, *Escherichia coli* was detected in 25 vegetable samples. *E. coli* O157:H7 was not found in samples, and 4 numbers (16%) of the isolates were non-O157 STEC. Among the isolates possess virulence genes, 40, 12 and 4% of them contained *eaeA*, *STx2* and both genes, respectively. But none of the isolates contained *STx1* gene (Table 2) (Figs 1- 4).

A total of 14 *E. coli* isolates possess virulence genes, were examined for antimicrobial resistance. The results showed that resistance against Tetracycline, Ciprofloxacin, Ampicillin, Cefotaxime, Trimetoprim-Sulfamethoxazol and Gentamicin were 57.14, 35.71, 57.14, 85.71, 57.14 and 100% respectively. (Table 3).

Table 3- Antimicrobial resistance of verotoxigenic *E. coli* isolated from vegetables.

| Antimicrobial Agent | Resistant | Intermediate | Sensitive |
|--------------------------------|-------------|--------------|------------|
| Tetracycline 30 | 8 (57.14%) | 2 (14.28%) | 4 (28.57%) |
| Ciprofloxacin 5 | 5 (35.71%) | 3 (21.42%) | 6 (42.85%) |
| Ampicillin 10 | 8 (57.14%) | 2 (14.28%) | 4 (28.57%) |
| Cefotaxime 30 | 12 (85.71%) | 1 (7.14%) | 1 (7.14%) |
| Gentamicin 10 | 14 (100%) | - | - |
| Trimetoprim-Sulfamethoxazol 20 | 8 (57.14%) | - | 6 (42.85%) |

Discussion and conclusion

In recent years, verotoxigenic *Escherichia coli* caused epidemics in some parts of the world and for this reason it has considered by researchers. Blanco reported that *E. coli* O157: H7 in level of 1 to 10 CFU/g may cause illnesses. It can survive for 15 days in lettuce (10). Also Bell et al. demonstrated that *E. coli* O157: H7 can be present in fruits such as apples (11). *E. coli* is not only regarded as an indicator of fecal contamination but more likely as an indicator of poor hygiene and sanitary practices during processing and further handling of food products. High prevalence of *E. coli* in milk was reported by Ali (63%), (12) and Lingathurai (70%), (13). The incidence of *E. coli* in food products is not significant because *E. coli* is normally an ubiquitous organism (14), but the pathogenic strains, if present could be harmful for consumers. In this study, from a total of 500 vegetable samples examined, 25 isolates of *E. coli* were identified; and none of the isolates were O157: H7. It could be due to the fact that in fall and winter the prevalence of serotype O157: H7 is lower than in spring and summer. So absence of serotype O157: H7 strains in vegetables examined in this study is acceptable.

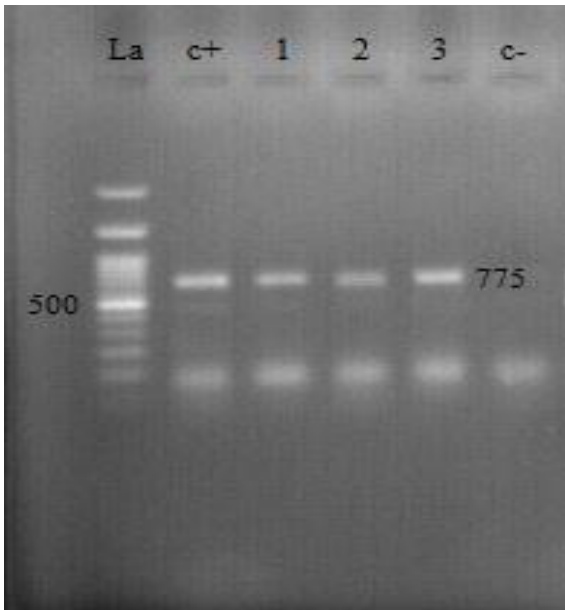


Fig 1- PCR for identifying *eaeA* gene (expected band: 775 bp) La: Ladder, C+: Positive control, C- : Negative control 1, 2 and 3: positive samples

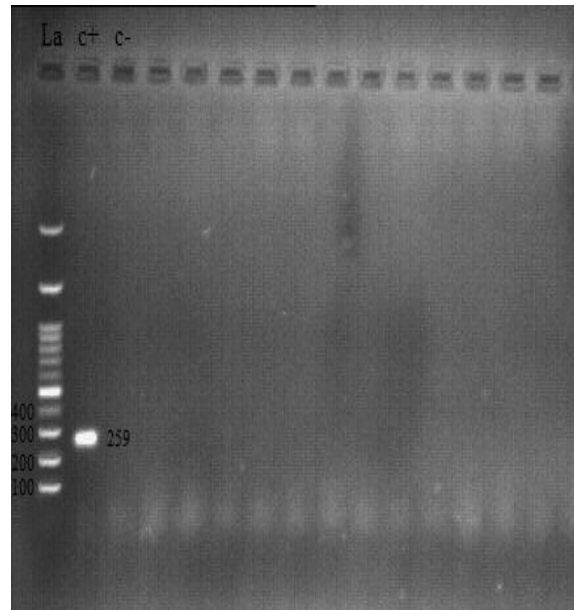


Fig 2- PCR for identifying *O157* gene (expected band: 259 bp) La: Ladder, C+: Positive control, C-: Negative control, No sample was positive

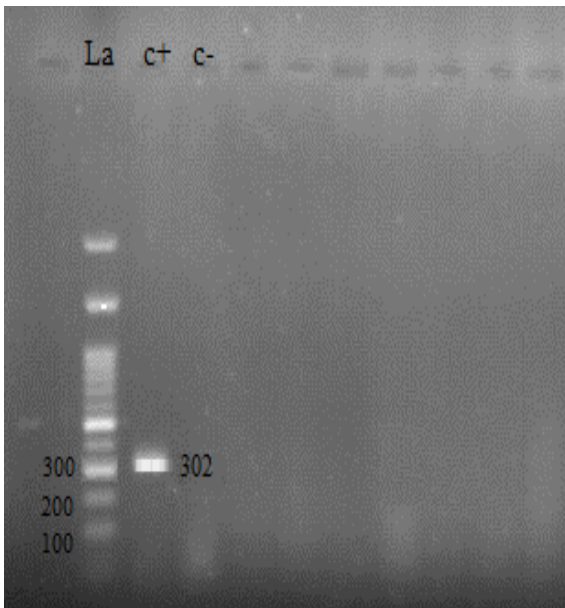


Fig 3- PCR for identifying *STx1* gene (expected band: 302 bp) La: Ladder, C+: Positive control, C-: Negative control. No sample was positive

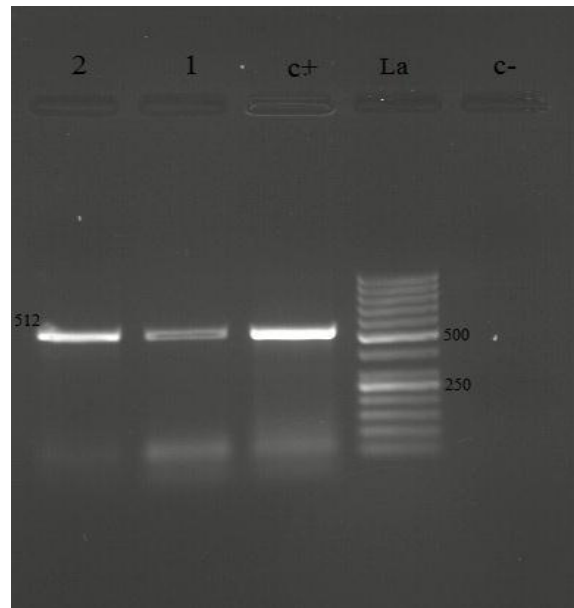


Fig. 4- PCR for identifying *STx2* gene (expected band: 512 bp) La: Ladder, C+: Positive control, C-: Negative control, 1, 2 positive samples

Unlike our results, in a study in Canada, 26 samples of unpasteurized Gouda cheese were tested and two samples were positive for *E. coli* O157: H7. This study was conducted due to incidence of HUS syndrome in Canada, in which 13 cases of HUS was observed. So consumption of cottage unpasteurized Gouda cheese was considered as the source of infection (15). In a study in Pennsylvania, from 248 milk samples tested for the presence of food-borne Pathogens, 4.2% of samples were positive for STEC (16).

In the present study, 5% of samples were contaminated with *E.coli*. This could be due to using human and animal manure as fertilizer which is a major source of this bacteria. Several studies indicated that *E. coli* can persist in manure-fertilized soil for several months (17 & 18). It is recommended to use compost manure for fertilizing organic Vegetables instead of human and animal manure (19). Practices of manure application have been shown to vary considerably among organic vegetable producers (20).

The results of the present study showed that none of the strains of *E.coli* possess genes *STx1*, *H7* and *rfbE*, but 40% of the isolates contain *eaeA* genes. Similarly, in a study in Spain on Shiga toxin-producing *Escherichia coli*, serotype O157: H7 strains isolated from tanks of fresh milk and cheese contained *eaeA* genes. In addition, it was found that 12% of isolates possess *STx2* gene and 4% contain both *eaeA* and *STx2* genes (21). Similarly, in a study by Garcia et al. in Mexico, from 22 STEC strains isolated from patients with diarrhea,

most strains harbored *Stx2* gene or combination of *eaeA* and *STx1* genes (22).

Based on the results of the present study, 85.71, 35.71, 57.14 and 100% of samples were resistant to cefotaxime, ciprofloxacin, trimetoprim-sulfamethoxazol and gentamycin respectively. In a study in Kermanshah-Iran, Mohajeri et al. found that from a total of 200 strains of *E. coli* 27, 5.22 and 26% of samples were resistant to cefotaxime, ceftaxidim, and ciprofloxacin, respectively (23). Many researchers reported the highest levels of resistance to those antimicrobials. About antimicrobial resistance of isolated strain in vegetables, highly variable resistance results were obtained and the resistance rate was quite high in some cases and low in others (24-27). However, in most cases, a direct comparison of studies is difficult because of different types of samples involved, different scopes of bacterial species targeted, different methods of strain isolation and different antimicrobials tested (28-30).

The presence of verotoxigenic *E.coli* in vegetables and high resistance of the isolates to antibiotics may be deduced from the results of this study. It could be a public health problem at the moment.

References

- (1) Gyles CL. Shiga toxin-producing *Escherichia coli*: an overview. *J Anim Sci* 2007; 85: 45- 62.
- (2) O'Brien AD., Tesh VL., Donohue-Rolfe A. Shiga toxin: biochemistry, genetics, mode of action, and role in pathogenesis. *Current Topics in Microbiology and Immunology* 1992; 180: 65-94.

- (3) Phillips AD., Navabpor S., Hicks S., Dougan A. Enterohaemorrhagic *Escherichia coli* O157:H7 targets Peyer's patches in man and causes attaching-effacing lesions in both human and bovine intestine. *Gut* 2000; 47: 377- 81.
- (4) Lindow SE., Brandl MT. Microbiology of the phyllosphere. *Applied and Environmental Microbiology* 2003; 69: 1875- 83.
- (5) Caprioli A., Morabito S., Brugere H. Enterohaemorrhagic *Escherichia coli*: emerging issues on virulence and modes of transmission. *Veterinary Research* 2005; 36: 289- 311.
- (6) Duffy G. Verocytotoxic *Escherichia coli* in animal feces, manures and slurries. *Journal of Applied Microbiology* 2003; 94 (Suppl.): 94-103.
- (7) Hepburn N F., MacRae M., Ogden ID. Survival of *Escherichia coli* O157 in abattoir waste products. *Letter in Applied Microbiology* 2002; 35: 233- 6.
- (8) Maule A. Survival of verocytotoxigenic *Escherichia coli* O157 in soil, water and on surfaces. *Applied Microbiology* 2000; 29: 71- 8.
- (9) Varnam AH. *Foodborne Pathogens*. London: Wolfe Publishing Ltd, 1991
- (10) Blanco M., Blanco JE., Mora A. Serotypes, virulence genes, and intimin types of Shiga toxin (verotoxin)-producing *Escherichia coli* isolates from healthy sheep in Spain. *Journal of Clinical Microbiology* 2003; 41: 1351- 65.
- (11) Bell C., Kyriakides A. Pathogenic *Escherichia coli*. in: Blackburn C., McClure P., eds, *Foodborne pathogens*. Cambridge 2000; 294- 5.
- (12) Ali AA., Abdelgadir WS. Incidence of quality of milk and milk products with special *Escherichia coli* in raw cow milk in Khartoum reference to *Salmonella* and its public health State British. *Journal of Dairy Sciences* 2011; 2 (1): 23- 6.
- (13) Lingathurai S., Vellathurai P. Bacteriological Quality and Safety of Raw Cow Milk in Madurai, South India. Available at: http://www.webmedcentral.com/article_view/1029, 2010.
- (14) Hahn G. Pathogenic bacteria in raw milk situation and significance. In: *Bacteriological quality of raw milk*. Brussels (Belgium). *International Dairy Federation* 1996; 67- 83.
- (15) Alves D., Kamali MA. 20-year population – based study of haemolytic syndrome: evidence for the importance of shiga- like toxin producing *E.coli*. *Recent Advance In VTEC Infractions. Infection and Immunity* 1999; 65 (7): 221- 34.
- (16) Jayarao BM., Donaldson SC., Straley BA. A survey of foodborn pathogens in bulk tank milk and raw milk consumption among farm families in Pennsylvania. *Journal of Dairy Sciences* 2006; 89: 2451- 8.
- (17) Ingham SC., Losinski JA., Andrews MP. *Escherichia coli* contamination of vegetables grown in soils fertilized with noncomposted bovine manure: garden-scale studies. *Applied and Environmental Microbiology* 2004; 70: 6420- 7.
- (18) Islam M., Doyle MP., Phatak SC. Persistence of enterohemorrhagic *Escherichia coli* O157:H7 in soil and on leaf lettuce and parsley grown in fields treated with contaminated manure composts or irrigation water. *Journal of Food Protection* 2004; 67: 1365- 70.
- (19) Anonymous. Soil fertility and crop nutrient management practice standard. National Organic Program. *United States Department of Agriculture*, Washington DC 2000; 205- 3.
- (20) Landau B., Koller M., Mader P. Pathogenic identification of critical control points for organic vegetable crops. Report Online Organic. Available at: <http://orgprints.org/20389/2013>.
- (21) Rey J., Sanchez S., Blanco JE. Prevalence, serotypes and virulence genes of shiga toxin-producing *E.coli* isolated from ovine and caprine milk and other dairy products in Spain. *International Journal of Food Microbiology* 2006; 107 (2): 207- 12.
- (22) Garcia T., Cerna JF., Paheco- Gil L. Drug resistant diarrheogenic *Escherichia coli*, Mexico. *Emerging Infectious Diseases* 2005; 11: 1306- 8.
- (23) Mohajeri P., Izadi B., Rezai M., Falahi B. Assessment of the frequency of extended spectrum Beta Lactamases producing *Escherichia coli* isolated from urinary tract infections and its antibiotic resistance pattern in Kermanshah. *Journal of Ardabil University of Medical Sciences* 2011; 11 (1): 86- 94. [in Persian]
- (24) Osterblad M., Pensala O., Peterzens M. Antimicrobial susceptibility of Enterobacteriaceae isolated from vegetables. *Journal of Antimicrobial Chemotherapy* 1999; 43: 503- 9.

- (25) Schwaiger K., Helmke K., Holzel CS. Antibiotic resistance in bacteria isolated from vegetables with regards (sic) to the marketing stage (farm vs. supermarket). *International Journal of Food Microbiology* 2011; 148: 191-6.
- (26) Hassan SA., Altalhi AD., Gherbawy YA. Bacterial load of fresh vegetables and their resistance to the currently used antibiotics in Saudi Arabia. *Foodborne Pathogen Diseases* 2011; 8: 1011- 18.
- (27) Holvoet K., Sampers I., Callens B. Moderate prevalence of antimicrobial resistance in *Escherichia coli* isolates from Lettuce, irrigation water, and soil. *Applied and Environmental Microbiology* 2013; 79 (21): 6677- 82.
- (28) McGowan LL., Jackson CR., Barrett JB. Prevalence and antimicrobial resistance of enterococci isolated from retail fruits, vegetables, and meats. *Journal of Food Protection* 2006; 69: 2976- 82.
- (29) Boehme S., Werner G., Klare I. Occurrence of antibiotic-resistant enterobacteria in agricultural foodstuffs. *Molecular Nutrition and Food Research* 2004; 48: 522- 31.
- (30) Johnston LM., Jaykus LA. Antimicrobial resistance of Enterococcus species isolated from produce. *Applied and Environmental Microbiology* 2004; 70: 3133- 7.

مقاومت آنتی‌بیوتیکی سویه‌های وروتوکسیژن باکتری *اشریشیا کلی* جدا شده از سبزیجات

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چکیده

مقدمه: عفونت‌های گوارشی ناشی از سویه‌های وروتوکسیژن باکتری *اشریشیا کلی* در دهه‌های اخیر شناسایی شده است. *اشریشیا کلی* O157:H7 مهمترین سروتیپ از این گروه است که موجب اسهال خونی و سندرم همولیتیک اورمیک در انسان می‌شود. این مطالعه به منظور شناسایی سویه‌های وروتوکسیژن باکتری *اشریشیا کلی* جدا شده از سبزیجات و مقاومت آنتی‌بیوتیکی آنها طراحی و اجرا شد.

مواد و روش‌ها: ۵۰۰ نمونه سبزیجات عرضه شده در شهرکرد به طور تصادفی از مغازه‌های خرده فروشی سطح شهر جمع آوری شد. آزمون‌های میکروبیولوژی و بیوشیمیایی به منظور جداسازی باکتری *اشریشیا کلی* روی نمونه‌ها انجام شد. آزمون زنجیره‌ای پلیمرز برای شناسایی ژن‌های *stx1*، *stx2*، *eae* و *rfbE* روی جدایه‌ها انجام و همچنین مقاومت آنتی‌بیوتیکی جدایه‌ها نسبت به آنتی‌بیوتیک‌ها به روش دیسک انتشاری ارزیابی شد.

نتایج: نتایج نشان داد که از میان ۲۵ باکتری *اشریشیا کلی* جدا شده ۴۰ درصد واجد ژن *eaeA*، ۱۲ درصد واجد ژن *STx2* و ۴ درصد واجد هر دو ژن بودند. هیچیک از جدایه‌ها واجد ژن‌های *H7 afbE* و *Stx1* نبودند. آزمون‌های آنتی‌بیوگرام نشانگر مقاومت بالای جدایه‌ها در برابر جنتامایسین و سفوتوکسیم بود.

بحث و نتیجه‌گیری: حضور سویه‌های وروتوکسیژن باکتری *اشریشیا کلی* در سبزیجات و مقاومت بالای جدایه‌ها در برابر آنتی‌بیوتیک‌ها می‌تواند یک خطر بالقوه برای بهداشت انسان باشد.

واژه‌های کلیدی: *اشریشیا کلی* وروتوکسیژن، ژن‌های حدت، مقاومت آنتی‌بیوتیکی، سبزیجات

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