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Estimation of virulence genes of Shiga toxin producing *Escherchia coli* from juice purchase and vegetables in Tehran/Iran

Reza Robati[1]* and Sakineh Gholami[2]

[1]*Department of Microbiology, Science and Research Branch, Islamic Azad University, Fars, Iran*
[2]*Department of Nursing, Islamic Azad University, Gachsaran Branch, Gachsaran, Iran*

ABSTRACT

The purpose of this study is isolation and identification of Escherchia coli 0157:H7 virulence genes from juice purchase and vegetables in Tehran. This cross-sectional study was performed on 200 samples of juice purchase and vegetables collected from Tehran. Isolates were enriched in ECB with novobiocin medium in temperature 37° C. After, in order to examine the fermentation of sorbitol and lactose the CT-SMAC and VRBA media and the activities of β-glucoronidase of separated bacteria were examined using the choromoagar medium. Then, the existence of E.coli 0157:H7 was confirmed with the specific antiserum. Finally, virulence genes stx1, stx2, eae and hly were tested by multiplex PCR and rfb O157 and fliC H7 were tested by single PCR. Out of all examined samples 156(78%) sorbitol negative bacteria were separated that after evaluation with specific antiserum 46 (23%) E.coli 0157:H7 was detected. The molecular markers, stx1 genes in 7 samples and eae, stx1 in 1 sample were detected. Intensive studies of the sources, incidence, fate and transport of E.coli 0157:H7 near produce production are required to determine the mechanisms of pre-harvest contamination and potential risks for humans.

Keywords: *E.coli* 0157:H7, Multiplex PCR, Virulence genes.

INTRODUCTION

STEC or Shiga toxin-producing *Escherichia coli* (*E.coli*) were first identified as a human pathogen in 1982. Since then, more than 200 serotypes of STEC have been isolated from animals, foods and other resources (Badouei 2011). *E.coli* O157:H7 are the most important serotypes of Shiga toxin-producing Escherichia coli that play a role in transmission of diseases by foods (Çadırcı 2010). This serotype is the main factor causing sporadic cases, outbreaks of hemorrhagic colitis and hemolytic uremic syndrome. Human infections caused by these bacteria are due to consumption of contaminated food and water (Mora 2007 & Grant 2011). Food products such as raw and unpasteurized milk, cheese, mayonnaise, fruit, vegetables, lettuce, salad, spinach, sprouts, radish, water, fruit, sausage, salami, hamburger and ground beef are reported as contamination sources of *E.coli* O157:H7 (Çadırcı 2010 & Grant 2011). Each microorganism requires a certain pH for its growth. Microorganisms are strongly affected by foods pH because have no special structure for regulation of their internal pH. Bacteria do not easily spoil foods with lower pH levels. Ability of *E.*coli O157:H7 to survive in low pH is well recognized. It can be survive in foods with low pH levels especially fruit juice and fermented sausages and salami (Small 2006). Fruits and vegetables sources of contamination include contaminated water and animal fertilizers used on agricultural land. When cattle

and other ruminants, such as deer may come in to farmland and vegetable fields, chances of contaminated fruits and vegetables with *E.*coli O157:H7 increases (Turutoglu 2007 & Wu 2008). STEC strains produce two powerful cytotoxins, called Shiga toxins (*Stx*1 and *Stx*2), which are encoded in the genome of prophages. These toxins have a cytopathic effect on intestinal epithelial cells that plays a role in the development of bloody diarrhea. STEC have other, additional virulence factors, the most important being a protein called intimin, which is responsible for both the intimate adhesion of bacteria to the intestinal epithelium and the attaching and effacing lesion of cell cup like structures. In enteropathogenic *Escherichia coli*, the *eae*A gene produces a 94-kDa outer membrane protein called intimin, which has been shown to be necessary but not sufficient to produce the attaching-and-effacing lesion. Intimin is encoded by the gene *eae* that plays a role in causing bloody diarrhea (Badouei 2011 & Liu 2011). Low infectious dose of bacteria necessities development of sensitive diagnostic techniques to ensure food safety (Fratamico 2010). Although the gene encoding Shiga toxin (*stx*1, *stx*2) and eae A/ *hly* genes are considered as major pathogenic genes of STEC strains, evaluation of *rfb* O157, H7 and *fli*C H7 in approved strains with antiserum can contribute to more accurate diagnosis of bacteria. Purpose of this study is to monitor strains of *E.*coli O157:H7 in samples of vegetables and traditional juice in Tehran and its comparison with serological and molecular methods for O157:H7 serotype in the isolated bacteria.

MATERIALS AND METHODS

A)Sampling: This cross-sectional descriptive study was conducted on 200 samples of Department stores offering products in four geographic regions of the West, East, North and South of Tehran in 2011. Information on geographical location, time of sampling and storage temperature were recorded in a questionnaire. Samples were transported to the microbiology laboratory based on cold chain and were tested immediately.

B) Enrichment: For purpose of enrichment, 25 g samples of lettuce and vegetables were isolated from juice samples They then cultured in 225 ml of EBC medium supplied by American Difco Company containing 20mg/l novobiocin supplied by American Sigma Company for 24 h at 37^0C in the stove.

C) Isolation of bacteria: All samples enriched on SMAC medium, supplied by Merc Company from Germany containing 0/05 ml/l Cefixim (Oxiod,UK) and 2/5 mg/l Potassium tellurite(Oxoid), were cultured for 24 h at 37^0C in the stove and Negative Sorbitol was purified. In order to evaluate lactose fermenting and identification of isolated bacteria, VRBA and EMB medium were used both of which were obtained by Merc Company. Moreover, for investigation of Beta Glucuronidase enzymes activity, some confirmed bacteria as Escherichia coli on special O157 medium were used to be cultivated for24 h at 37^0C in the stove which were obtained from Haimdia Company from India (Vimont 2006).

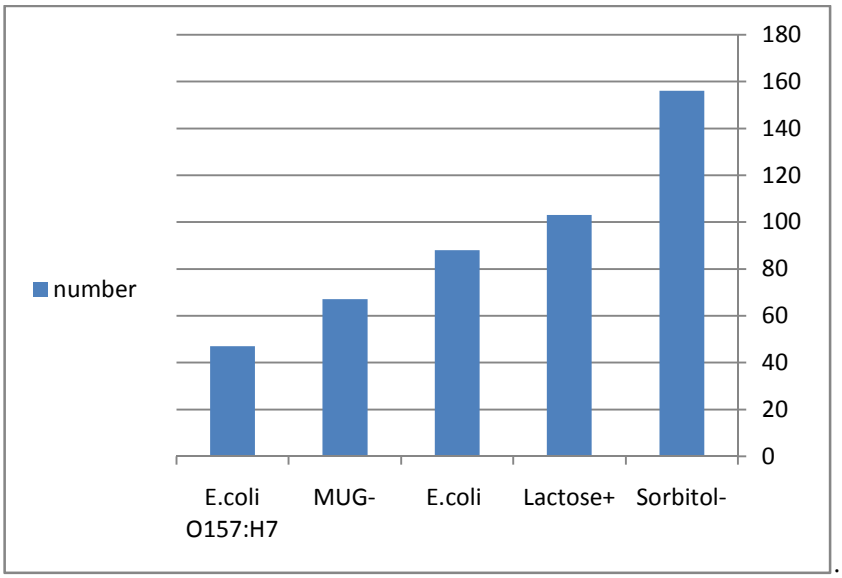
D): Serotyping: For final confirmation, Sorbitol-Negative colonies and Beta Glucuronidase were used obtained by agglutination test with specific O157 antiserum (Liu 2011).

E) Assessment of of pathogenic genes: DNA was extracted using a DNP ^{TM}kit (Sinagene, Iran). For simultaneous detection of pathogenic genes such as *hly*, *eae* A, *stx*2, *stx*1, the Multiplex PCR introduced by Sanataniello in 2007 was used. PCR final volume of the reaction was 50 micro liters containing Tris-HCL (10 mM)، (0.2 mM) dNTPs، (3 mM) mgCl2، 20 Pmol، (10Mm) KCl Of each primer, 1 unit of DNA enzyme and DNA 4 µl. PCR was performed using Thermocycler Equipment (Techne, UK) with temperature conditions as follows: Temperature of 95 ° C for 3 min(initial Denaturation), Temperature of 95 ° C for 20 seconds (Denaturation), Temperature of 58 ° C for 40 seconds (Annealing), Temperature of 72 ° C for 90 seconds (Extension), Temperature of 72 ° C for 5 min(Final Extension). Finally, 10 ml of PCR product was put on %1/5 agarose gel and transferred followed by ethidium bromide and was studied after electrophoresis by As a positive control Transilluminator . It was used as positive control of *E.coli* K 933O157:H7 strain and a negative control strain of *E.Coli* k12 (Kim 2005). Gene-specific primers of *rfb* O157 and *fli*C H7 were used to determine exact identification of O157:H7 (Badouei 2010).

RESULTS

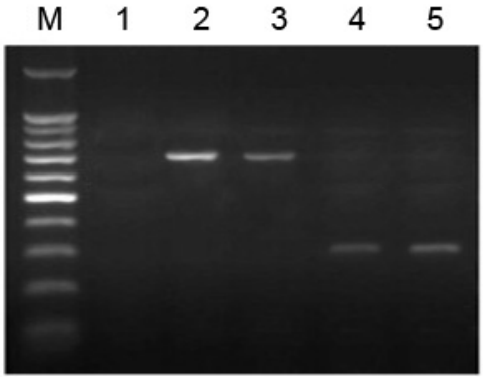
Among collected samples, 45 vegetables (%22/5), 56 radishes(%28), 10 spinach(%5), 9 lettuce(%4/5), 80carrots(%40) were available. Among the total samples in the CT-SMAC medium, 156 negative sorbitol colonies(%78) and 103 lactose-positive colonies were isolated in VRBA medium. After cultivating on specific media, 88 samples (%44) were identified as *Escherichia coli*. After examining the activity of Beta Glucuronidase on

CHROMagar, 67 negative *Escherichia coli* beta glucuronidase(MUG)(%33/5) were isolated and 47 *E.coli* O157 bacteria (%23/5) were isolated(diagram 1).

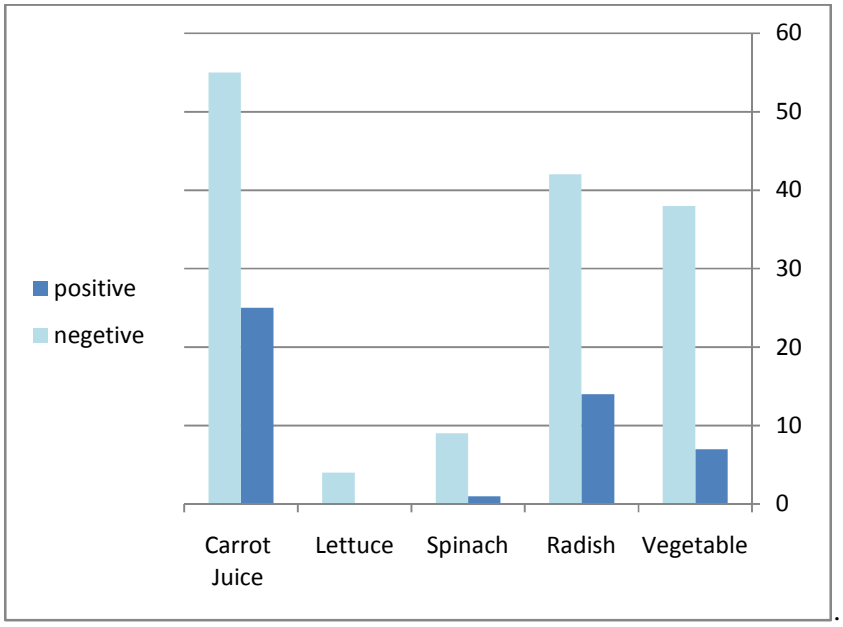


(Diagram1) Distribution and abundance of bacteria suspected to O157:H7 based on biochemical tests

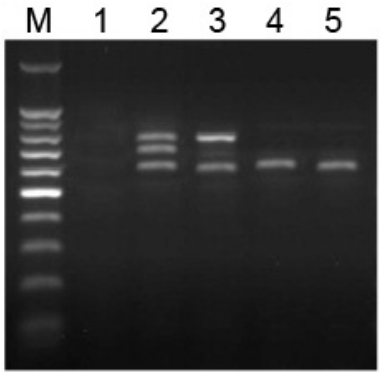
Most serotypes identified by serological methods were related to carrot juice and the least one was in spinach with frequencies of %52/38 and %2.13(diagram2), respectively. Following molecular analysis of total confirmed samples by antiserum, 38 samples of (%80/85) *rfb* O157 gene and 20 (%42/55) samples of *fli*C H7 genes were available(figure 1). Moreover, in seven samples of stx1 gene(%3/5) an in three sample of *stx*1 and eaegenes(%1/5), it was observed that all 9 cases were related to strains confirmed with *rfb* O157 and *fli*C H7 genes as *E.coli* O157(figure2).



(Figure1) View of serotype-specific genes in *E*.coli O157:H7 on agarose gel. M line: Size of 100 bp marker, 1 line: Negative control, 2 & 3 lines are Samples containing *fli*C H7 genes and 4 & 5 lines are Samples containing rfb O157



(Diagram2) Absolute and relative frequency of bacteria isolated with serological methods based on the type of sample.



(Figure2) View of pathogenic genes determined by Multiplex PCR method By specific primers on agarose gel.
M line: Size of 100 bp marker, 1 line: Negative control, line 2 positive control, line 3 is Sample containing two eae & stx1 genes and 4 & 5 lines are Samples containing stx1 genes.

DISCUSSION

Escherichia coli O157: H7 is the fourth pathogenic factor of food-borne disease in United States. Number of cases associated with consumption of raw fruit and vegetables has increased over recent years (Koohmaraie 2007). Due to the long term bacteria survival in the environment, there is possibility of indirect transfer or displacement of bacteria from insects. Because insects are in contact with fertilizer and manure, they may cause the disease distribution. Slug animals such as snail are agricultural pests that can contaminate leafy vegetables. These animals are constantly engulf bacteria in soil and their environment. Therefore, they have contamination potential with 0157 Strain. Invertebrates may play a role in transmission of 0157 strain to fruit and vegetable via direct contact with stool contamination. Various studies have shown that Escherichia coli most often hide in sides and gaps of vegetable and fruit or in nutritional place of slug. These areas not only protect the bacteria against factors such as washing and cleansers, but also are considered as places for contamination transmission (Sprostom 2006).

Between 1990 and 2000, juice (including cider and apple juice) and products like alfalfa sprouts, lettuce, and cabbage created widespread outbreaks in different parts of the world. The most famous case is related to Japan in 1996 following consumption of radish sprouts (Kodaka 2004).

In the United States, consumption of fruit and raw vegetables was related to outbreaks caused by these bacteria. In 2005 and 2006, an outbreak associated with consumption of packaged lettuce and a larger outbreak associated with spinach were reported respectively. Isolated strains in both outbreaks were *E.coli* O157:H7 (Cooley 2007).

Results of this study indicated that among 47 confirmed bacteria with antiserum, %2/13, %14/92, %29/57, %53/38 were related to spinach, vegetable, radish, and carrot juice, respectively. The result was predictable, given the fact that most collected samples were related to carrot juice with probability of carrots contamination in farm fields during harvest and due to lack of proper cleaning.

The results of several studies showed improved survival of *E.coli* O157:H7 in clay soils, and reinforced soils with manure near the roots of plants. Also considering bacteria survival in the ground for 4 to 8 weeks without emission in the environment, minimum 120 days interval is suggested between using animal manure and harvesting (Cooley 2007).

Alam and colleagues in 2004 in America, investigated *E.coli* O157:H7 prevalence and its relationship with home flies to see better its ecology. The above researchers collected 3440 home flies in 2 cattle breeding farms over a period of 4 months. All the samples were investigated in terms of contamination with the bacteria. Prevalence of bacteria in flies collected from the manger and animal feed stores were estimated %2/9 and %1/4, respectively. In addition, contamination of infected flies reported as $1/5\times10^5$ - 3×10^5CFU. *eae*، *stx*2، *stx*1 and *fli*C genes were isolated using PCR method in *E.coli* O157:H7 and positive results were reported as % 90/4, %92/2 and %100, respectively. Large population of flies on cattle farms are considered as *E.coli* O157:H7 emission factor among animals and their environment (Alam 2004).

Blanco and colleagues in Spain in 2003 used Mutiplex PCR to investigate، *stx*2، *stx*1 and they used PCR for separate detection of eae and hly genes. Among total 384 detected strains of STEC, *stx*2, *stx*1, *eae* and *hly* genes were expresses as 55%, 3%,42%, 6% and 28%, respectively. High frequency of *stx*1 gene was considerable (Blanco 2003).

Osek(2003) in Poland in an study investigated existence of pathogenic *stx*2, *stx*1(along with its variants) , *eae* A, *hly* A, *rfb* O157, *fli*C H7 genes in bacteria isolated from children samples and bovine stool samples. Results of this study indicated a 12/4% frequency in Shiga-toxin genes. Among these 25 strains with *stx* genes, 6 isolates of *E.coli* O157:H7 serotype and 4 isolates of *E.coli* O157:NM were available, respectively. In 20 STEC strains, *eae* A, *hly* A genes were also detected (Osek 2003).

Uhlich et al, in 2006 reported outbreaks in United States following consumption of spinach and lettuce infected by *E.coli* O157:H7. strains isolated in this outbreak were closely related genetically and after performance of PCR, all strains of *eae*, *wyzo*157 and hly genes/ one of *stx*2, *stx*1 genes or both of them were mentioned (Uhlich 2008).

Fratamico in 2010 in United States used Multiplex Real-Time PCR to detect *E.coli* O157:NM(fixed NM / H-) in food samples. He conducted his study using the gene-specific primers for study of *wyzo*157, *stx*2, *stx*1, *fli*C H6 and *eae* in isolated strains.

In this study, we used Multiplex PCR method in specific confirmed samples of antiserum to detect and *eae* A genes in isolated strains. Among three strains isolates with *stx*l gene and one strain with stxl and eae A genes were available (Çadırcı 2010).

Risks of infection with *E.coli* O157:H7 and experiences related to contamination with this bacteria indicates the importance of exact tests on detection of O157:H7 serotype. If quick identification of bacteria occurs, then source of its outbreak would be sooner detected and treated. Simultaneous molecular analysis on environmental samples (like water and soil) and vegetables may better indicate genetic relationship between strains and also etiology of the pathogen (Cooley 2007).

Due to the long term survival of these organisms in the soil and environments with low pH level, risk of contamination of food, especially the fruit fallen the tree increases. Therefore, there is a need for more care and prevention at harvesting. Methods that focus on reducing E. coli O157:H7 population before entering human food chain have important role in reduction of Human Diseases (Callaway 2003).

CONCLUSION

Considering low infectious dose and possibility of vegetables and fruits contamination with *E.coli* O157:H7, it is necessary to control foods during different stages of planting, harvesting it's and supplying to the market.

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