Pharmaceutical Sciences
September 2016, 22, 195-202
doi: 10.15171/PS.2016.31
http://journals.tbzmed.ac.ir/PHARM

Research Article

Occurrence, Molecular Detection and Antibiotic Resistance Profile of *Escherichia coli* O157:H7 Isolated from Ready-to-Eat Vegetable Salads in Iran

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Article Info

Article History:
Received: 19 January 2016
Accepted: 10 May 2016
ePublished: 30 September 2016

Keywords:
-Ready-to-eat vegetable salads
-Antibiotic resistant
-*Escherichia coli* O157:H7
-Tabriz
-Real-time PCR

ABSTRACT

Background: *Escherichia coli* O157:H7 is one of the high risk bacteria, found in foods, especially in ready-to-eat products, which causes many disorders such as hemorrhagic colitis, hemolytic uremic syndrome and dysentery in humans. The objective of this study was to determine the contamination of *Escherichia coli* O157:H7 and antibiotic resistance in ready-to-eat vegetable salads.

Methods: In this study, 6o ready-to-eat salad vegetable samples were collected from restaurants for determining their isolated *Escherichia coli* O157:H7 and their antibiotic resistance by SYBR Green I Based real-time PCR and disc diffusion method, respectively.

Results: Out of the 60 samples, 10 samples (16.66%) were positive for *Escherichia coli* O157:H7. All identified isolates were resistant to more than five antibiotics. A high rate of resistance was observed to the clindamycin, vancomycin, erythromycin, penicillin, amoxicillin and cephalothin (100%), cefixime (40%), amikacin (20%), cefotaxime and cetracycline (10%).

Conclusion: The presence of toxigenic *Escherichia coli* O157:H7 in ready-to-eat vegetable salads can be considered a public health threat. Providing the useful strategies for improving food safety and inspection services is necessary. Further studies are suggested to identify specific ready-to-eat vegetable salads-related hazards.

Introduction

Recently, high tendency to the consumption of ready-to-eat foods has been reported as one of the obvious changes in the human life style. Salads containing fruits and vegetables are considered as a main part of healthy diet.[1,2] Due to the presence of vitamins, minerals, fiber, prebiotic and antioxidants in fruits and vegetables, they are frequently used for improving lipid profile disorders and oxidative stress, protecting against chronic diseases such as cardiovascular disease, diabetes and many types of cancers as well as other malignancies.[1,3-5] Food contamination with food-borne pathogens and toxin is an emerging public health hazard that is considered as current context of food safety in Iran.[6] It seems that providing safe and healthy ready-to-eat vegetable salads (REVS) could be a good strategy for protection against these diseases. However, salad vegetables could be contaminated with pathogenic microorganisms during growth and breeding (primary contamination), or washing, slicing, soaking, packaging and preparation process (secondary contamination).[7,8] The consumption of raw vegetables plays an important role in the transmission of human pathogens such as *E. coli* O157:H7.[9,10] According to the Zoonosis report, consumption of raw vegetables contaminated with enteric pathogens was involved in prevalence of 4.4% foodborne illness in the EU and United States. According to the Scharff report, these diseases had an annual cost of $ 38,593 million in the United States in 2010.[1]

E. coli O157:H7 is a very malignant pathogenic bacterium that produces one or more types of

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cytotoxins known as shigatoxin (Stx) or verocytotoxin. The toxins have been implicated in a large number of foodborne diseases. This toxin can cause many diseases such as hemorrhagic colitis, hemolytic uremic syndrome (HUS), mild to severe diarrhea, thrombotic thrombocytopenic purpura or death especially in children below 10 years of age.[11,12] In recent years, the contamination of vegetables by *E. coli* O157:H7 has increased and is regarded as a major cause of mortality and morbidity.[11,13] The existence of *E. coli* O157:H7 in the contaminated vegetables sprouts and fresh juice has been reported from Europe, North America and Japan.[14] In Argentina, the first reported cases of foodborne outbreaks, associated with ready-to-eat salads, were contaminated with *E. coli* O157:H7.[15]
There are several methods for the identification of pathogenic bacteria in foods, including culture media, ELISA and PCR. Basically the culture methods require several days to recognize the pathogenic bacteria and to confirm the suspected colonies by biochemical and serological methods (5–7 days).[16,17] The detection of other pathogens can also be time-consuming using conventional methods. In recent decades molecular methods such as real-time PCR (q-PCR) have been used due to their rapid and sensitive detection of pathogenic bacteria in food products and their high accuracy in detecting the presence of a single type of pathogen in food samples.[18,19]
According to the previous studies, antibiotics are used increasingly for preventing bacterial infection mortalities worldwide.[20,21] The emergence of antimicrobial resistance in bacteria such as *E. coli* is another public health hazard.[22] However, studies indicate that some antibiotics, if used at the beginning of infection, may reduce the severity of the disease.[23] There is little data about the prevalence of *E. coli* contamination in REVS. In addition, we have limited knowledge on antibiotic resistance characterization of *E. coli* isolated from these types of products in Iran. Therefore this study was carried out to evaluate the contamination load and antimicrobial resistance in REVS in order to obtain data for quantitative risk assessments of *E. coli* O157:H7 in this type of foods in Tabriz, Iran.

Material and Methods

Location

Tabriz is the fifth largest city in Iran. Located in the north-west of the country, the city has a population of more than 1.6 million people (2014) in an area of 45,481 km^2.

Restaurants

This was an observational study, since we were not officially permitted to inspect the restaurants. We asked unofficial questions from the restaurants in relation to the contamination of vegetables served in them. REVS samples were purchased in the city of Tabriz, in 5 areas categorized as: North (N), South (S), West (W), East (E) and Central (C).

Determination of sample size

Sample size was determined using the following formula, based on the reported pollution percentage of *E. coli* O157 in salad vegetables, which was estimated at 0.69 following Abdi et al.[24]

$$n = \frac{z^2 p(1-p)}{d^2} \qquad \text{Eq.(1)}$$

Z=1.96, P=0.69(contamination Percentage), d=0.12
The confidence level and the precision were 95% and 0.12, respectively. Thus, the required sample size was estimated 58; however, 60 samples were taken deliberately in order to maximize the precision of the study.

Sampling and preparation

The samples were 60 packaged ready-to-eat vegetable salads (REVS) purchased from 5 areas from February to June 2015. They were tested for the presence of Escherichia coli O157:H7. The ingredients of the salads obtained from all the restaurants were similar. They were mixed salads containing raw vegetables such as lettuce, cabbage, tomato, cucumber, and carrot that had been prepared by the restaurants. The average weight of each REVS sample at the time of purchase was approximately 100 g. Samples were placed in sterilized plastic bags and were transported to laboratory and stored in a refrigerated container at 4°C until sample preparation and analysis.
From each REVS sample, 50 g of its representative portion was transferred into a sterile plastic pouch, containing 450 ml of 0.1% sterile peptone water homogenized for 60s using a pulsifier (made in UK) at room temperature. The precipitate was centrifuged for five minutes at 8,000 rpm in sterile tubes. The precipitate was washed three times with 0.1% sterile peptone water and the centrifugation procedure was repeated. In the last stage, peptone water was added to the precipitate which was then stored at -20°C until it was used for DNA extraction.

DNA extraction

DNA extraction was performed using Accuprep Genomic Kit (Bioneer, South Korea) according to the manufacturer's instructions with some modifications as follows:
Briefly, 500 µl from each REVS samples plus 200ml of Tissue Lysis buffer was placed in a sterile micro tube. Then, 30 µl of proteinase K was added, mixed and incubated at 60 °C for 1 hr. By adding 200 µl of binding buffer, the mixture was immediately mixed and incubated at 60 °C for 10 min and then was thoroughly mixed with 100 µl of

Table 1. Primers used for the detection of E.coli O157:H7

Organisms	Target gene	Primer sequence (5′–3′)	Condition	Product (pb)	Reference
E.coli O157:H7	eaeA	CCAGTATCGCGACTGTCGATG ACTCCAGAACGCTGCTCACT	95°C 40S, 60°C 40S 72°C 40S, 40 Cycle	316	this study

Isopropanol by pipetting. The content of the tubes was transferred into a new tube with silica filter. The tube was centrifuged at 12,000 rpm for 2 min and then the flowthrough was discarded. The tube was washed two times with ethanol 70% and 95%, respectively. The tube was air-dried by centrifugation at 13000 rpm for 2 min. The bound DNA was eluted by 100 ml Tris-EDTA buffer. The extracted DNA was stored at -20 °C until it was used for Real-time PCR. The extracted DNA was assessed qualitatively and quantitatively by gel electrophoresis and Bio photometer, respectively (Eppendrof, Bio photometer plus Germany).

SYBR Green I Real time PCR

SYBR Green I Real time PCR was performed using Rotor GeneQ- 5 PLEX, (QIAGen, Germany). In 10 µL volume of reaction there were 5µL 2x SYBR Green I master mix (Bioneer, South Korea), 0.5 µL of each primer, 1 µL template DNA and 3 µL H2O. The oligonucleotide sequences for each primer and PCR thermal conditions for detection of E.coli O157:H7 are shown in Table 1.

Genomic DNA of E.coli O157:H7 was used for preparation of the standard curve. Liquid culture of E.coli O157:H7 strain/ATCC43890 was used for DNA extraction and for spiking experiments of REVS. DNA was extracted from a dilution series with concentrations ranging from 10^1 to 10^8 CFU/ml in 10-fold dilutions of E. coli O157:H7, grown overnight in 0.1% peptone water while non-spiked samples were used as negative control.

Real time PCR and analysis were performed in Rotor Gene Q- 5 PLEX, (QIAGen, Germany). In SYBER Green I Real time PCR, the amplification of the DNA target was measured in terms of the increment in the quantity of fluorescent which was determined at the end of each amplification cycle. In brief, SYBR Green I bind with the minor groove of double strand increased fluorescence greatly. The fractional cycle in which the increase in fluorescence was generated by the accumulation exceeded 10 standard deviation of the mean base line fluorescence, with a selected range of cycle referred to as the threshold cycle (CT).

The specificity of the reaction was given by the Tm of the amplicons immediately after the last cycle which is known as melting curve. This process consisted of slow heating of amplicons from 65 to 95°C with a ramp of 1°C/min. During this time, a rapid decrease in fluorescence occurred due to the denaturation of the amplicons, which resulted in the formation of single strand of DNA and detachment of SYBR Green I. For obtaining the optimal concentration of primers, preliminary tests were performed using 200, 100, 50 and 25 pmol/ml concentrations of the forward and reverse primers.

Antibiotic resistance of Escherichia coli O157:H7

For evaluating the resistance of antibiotics to the Escherichia coli O157:H7 isolates from the REVS samples a disk was used as standard agar disc in diffusion method in Mueller–Hinton Agar (QUELAB UK) with 14 antibiotics. The 14 antibiotic disks included chloramphenicol (30µg), cefixime (5µg), tetracycline (30µg), ciprofloxacin (5µg), amikacin (30µg), nalidixic Acid (30µg), clindamycin (2µg), vancomycin (30µg), erythromycin (15µg), penicillin (10µg), amoxicilin (25µg), cefotaxime (30µg), kanamycim (30µg) and cephalothin (30µg). The antibiotic disks were obtained from PADTAN TEB (IRAN). Resistance was estimated by measuring CLSI zone diameter using interpretive standards. Discs were categorized as resistant, intermediate or susceptible.

Results

Standard Curve

The standard curve plotted using the C_T of various concentrations of Escherichia coli O157:H7 (ranging from 10^1 to 10^8 CFU/ml) showed a good linear with R^2 of 0.9966 (Figure 1). The sensitively of the test was less than 10^2 CFU/ml.

Microbiological quality of REVS samples

In this study, 60 REVS samples were purchased from restaurants of Tabriz to evaluate the risk of infection by Escherichia coli O157:H7 using SYBR Green I Real-time PCR. The obtained results showed 10 (16.66%) samples were positive to Escherichia coli O157:H7 contamination. These positive samples were identified in East, Center and North areas. The highest contamination rate of Escherichia coli O157:H7 isolates was 6 (42.85%) of the 14 REVS in East, 3 (30%) of the 10 REVS samples in Center and 1 (10%) of the 10 REVS in North. Table 2 shows the results of SYBR Green I qPCR method for *E. coli* O157:H7.

The Mueller–Hinton Agar of 14 antibiotic-resistance of *E. coli* O157:H7, identified from 10 probable isolates of 60 REVS samples, was analyzed (Figure 2). All (100%) identified isolates were resistant to more than five antibiotics. High rates of resistance were observed for clindamycin (100%), vancomycin (100%), erythromycin

(100%), penicillin (100%), amoxicillin (100%), cephalothin (100%), cefixime (40%), amikacin (20%), cefotaxime (10%) and Tetracycline (10%). The resistance of isolates to the antibiotics was presented in Table 3.

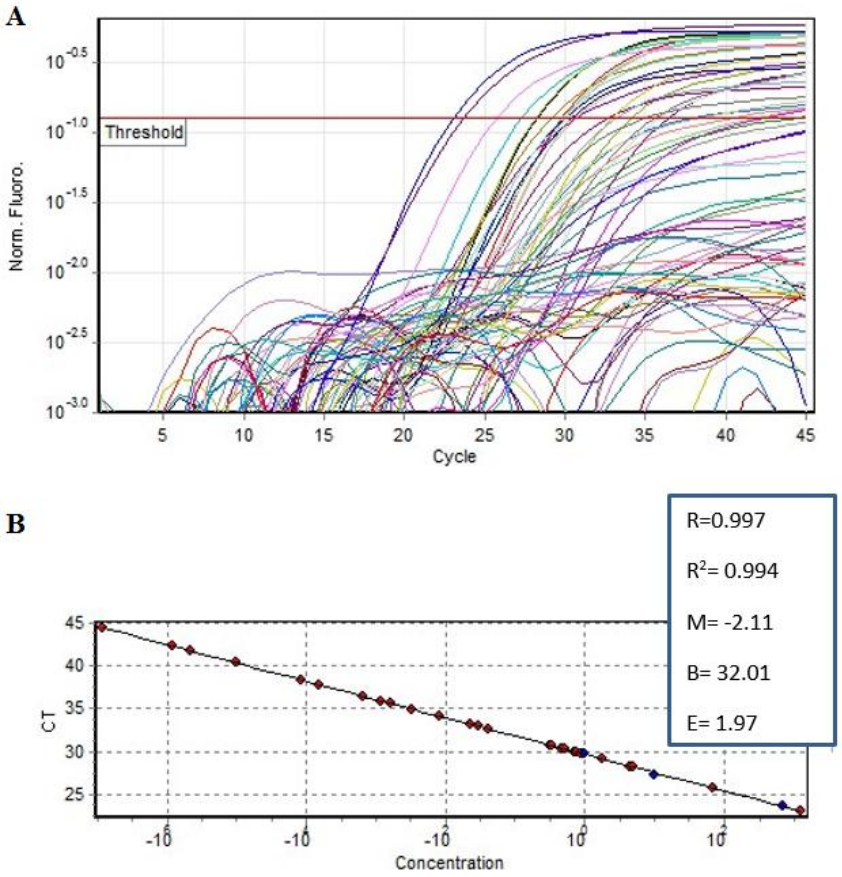

Figure 1. A: The specificity of the SYBR Green I real time RT-PCR assay of detection of Escherichia coli O157:H7 REVS samples.
B. Standard curve showing a linear relationship between standard DNA concentrations and CT values. A linear regression curve was generated by the CT means.

Figure 2. Antibiotic-resistance analysis of the Mueller–Hinton Agar for *E. coli* O157:H7.

Table 2. Prevalence of *E. coli* O157:H7 isolated from REVS by SYBR Green I qPCR method.

Area type	No. of samples	Contaminated Samples	Number of positive samples (%)
North	10	N_2	1(10)
West	10	-	0(0.00)
Central	10	C_2	3(30)
		C_3	
		C_4	
East	14	E_5	6(42.85)
		E_6	
		E_9	
		E_{10}	
		E_{12}	
		E_{13}	
South	16	-	0(0.00)
Total	60	-	10(16.66)

Table 3. Antibiotic resistance of *E. coli* O157:H7 isolated from REVS.

Class and antimicrobial	No. of isolates (n=10)		
	Resistant	Intermediate	Susceptible
β-Lactams			
CTX	1	7	2
P	10	0	0
AMX	10	0	0
CFM	4	0	6
CF	10	0	0
Phenicols			
C	0	3	7
Tetracyclines			
TE	1	0	9
Quinolones			
NA	0	2	8
CP	0	0	10
Aminoglycosides			
K	0	9	1
AM	2	2	6
Macrolides			
E	10	0	0
Glycopeptide			
V	10	0	0
Lincomycin			
CC	10	0	0

CTX: Cefotaxime, P: Penicillin, AMX: Amoxicillin, CFM: Cefixime, CF: Cephalothin, C: Chloramphenicol, TE: Tetracycline, NA: Nalidixic Acid, CP: Ciprofloxacin, K: kanamycin, AM: Amikacin, E: Erythromycin, V: Vancomycin, CC: Clindamycin

Discussion

This is the first report on the microbiological quality of REVS conducted in Iran. This study showed among REVS samples, 10 (16.66%) of 60 samples had a low microbiological quality. The frequency of contamination with *E. coli* O157: H7 was higher than those reported for REVS in other countries. For example, prevalence of 5%, 4% and 1.3% of *E. coli* contamination were reported in studies from Turkey,[24] Portugal[2] and United Kingdom,[25] respectively. This contamination may be associated with other factors such as exposure to contaminated water/soil, animal manures or preparation and personal hygiene.[26,27] Furthermore, in this study, no contamination with *E. Coli* O157:H7 was observed in the REVS collected from the west and south parts of the city. Annual visits of health experts to the south and west parts of the city were more than the other parts of the city (Data not shown). This may be the reason for the low level of pollution in these parts. Contamination of REVS by Escherichia coli O157: H7 is considered

a major public health problem because of the high incidence, the severity of the diseases and high mortality rate.[28] Thus, assessing the quality of vegetable sources and the overall hygienic status of the restaurants are necessary.[26]According to the latest report by the EFSA (EU) and CDC (UUEE) incidence rates per 100,000 people were 0.7 and 0.99 for enterotoxigenic *E. coli* (STEC), respectively. The mortality rate was 1.5% for STEC. [28] In addition, six cases of illness by *E. coli* O157:H7 were reported by the Public Health Agency of Canada (PHAC).[29] Recent studies in less developed countries reported human pathogenic bacteria in ready-to-eat salads with low microbiological quality. Thus, the findings emphasize the importance of diagnosis and prevention of these diseases.[2] For this reason, it is absolutely necessary to develop rapid and sensitive methods for routine microbiological determination of foods in order to ensure the safety of products. Molecular methods such as PCR have widely been used to identify foodborne pathogens i.e. *E. coli* O157:H7 in ready-to-eat vegetables salads. qPCR technique is rapid, sensitive, more reliable and accurate in determining pathogenic bacteria. The main advantages of the qPCR molecular beacon-based foodborne pathogen detection under routine conditions are the high specificity and that not more than 28 h for *E. coli* O157:H7. This is a very important factor for determination of contamination load in REVS samples that cannot be stored for a long time. The application of qPCR is a great alternative to cultural procedure and assistance in sketching more impressive control programs as it permits a high throughput of samples.[16]

Salad vegetables may be contaminated with antimicrobial-resistant bacteria through irrigation water, during processing and handling.[22] Resistant bacteria, such as multiple-drug (including amoxicillin) resistant *E. coli* presents a serious problem worldwide.[2] In Europe, there have been many reports of illness, death and clinical infections caused by resistant bacteria, including the multi-resistant *E. coli* the source of which was water-sprayed vegetables. One report of bloody diarrhea and hemolytic uremic syndrome in Germany resulted in thousands of people being infected with HUS 877 (with 32 deaths) and 3,043 cases of EHEC (with 16 deaths).[20] The increase and emergence of the antibiotic resistance of microorganisms is probably due to increased use of antibiotics in the last decades.[30-32]

Conclusion

Despite recent evidence indicates[3-5,33] that a high consumption of plant-based foods such as fruit, vegetables and salads is associated with a low risk of chronic disease. However, salads remain a public health risk for the transmission of antibiotic-resistant E. coli O157:H7. Thus, appropriate washing to reduce contamination of these foods is recommended.

Acknowledgments

The authors would like to thank the restaurant owners. The Nutrition Faculty and the Vice Chancellor of Research of Tabriz University of Medical Sciences, Iran, financially supported this research. This article was written on the basis of data obtained for an MS thesis about food sciences.

Conflict of interests

The authors claim that there is no conflict of interest.

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