Received Date : 21-Jan-2013
Revised Date : 09-Jun-2013
Accepted Date : 14-Jun-2013
Article type : Original Article

Short running head: Pathogenic *E. coli* in Vegetable Samples in Istanbul

# Evaluation of Pathogenic *Escherichia coli* Occurrence in Vegetable Samples from District Bazaars in İstanbul Using Real-Time PCR

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## SIGNIFICANCE AND IMPACT OF THE STUDY

We assessed the occurrence of virulent *Escherichia* (*E.*) *coli* and Shiga-toxin-producing *E. coli* (STEC) virulent populations in the vegetable samples collected from several

district bazaars in Istanbul, Turkey. The results indicated that the vegetables from the bazaars had poor microbial quality and represented a potential health risk for customers.

## ABSTRACT

In this study, a total of 180 vegetable samples collected from several district bazaars of Istanbul were investigated for the occurrence of *Escherichia coli* using a culture-based method. The isolates were subjected to real-time PCR detection of Shiga toxin-producing *E. coli* (STEC) using primers specific for the Shiga toxin (*stx1* and *stx2*) and intimin (*eae*) virulence genes. The prevalences of *E. coli* in the samples were 93.3% in spinach, 93.3% in lettuce, 86.6% in parsley, 43.3% in carrot, 33.3% in cucumber and 13.3% in tomato. Of 180 samples, 13 contained STEC (six parsley, three carrots, three lettuces, and one cucumber out of 30 samples of each). Among 13 STEC-positive isolates, presence of *stx1*, *stx2*, and *eae* were detected in only one sample; *stx2*, and *eae* in two samples, and *stx2* in ten samples. Serotype O157 was found in parsley, lettuce and carrot; O26 in lettuce, parsley, cucumber and carrot; and O111 and O113 in parsley only. In conclusion, STEC was present in vegetable samples marketed in several district bazaars in Istanbul; this might represent a route of transmission of pathogenic STEC to humans and be harmful to public health.

Keywords: Shiga toxin-producing *Escherichia coli*, vegetable, public health, serotypes, real-time PCR

## INTRODUCTION

Shiga toxin-producing *Escherichia coli* (STEC), also known as verotoxin-producing *E. coli* (VTEC) or enterohemorrhagic *E. coli* (EHEC), is one of the most important

foodborne pathogens that have emerged within the past two decades. This pathogen is linked worldwide to severe diseases and complications that cause human morbidity and mortality, such as bloody and non-bloody diarrhoea, haemorrhagic colitis (HC), and Haemolytic Uraemic Syndrome (HUS) (Griffin and Tauxe, 1991; Beutin *et al*. 2004; Pennington 2010). Shiga toxins, also known as verotoxins, contribute to the pathogenicity and are encoded by the *stx1* and *stx2* genes, each of which exists as several variants. Intimin is another virulence factor that is encoded by the *eae* gene and mediates tight attachment of bacteria to enterocytes (Ito *et al*. 2007). More than 200 distinct STEC serotypes associated with human diseases are recognized, including O157 and a number of non-O157 STEC serotypes (Zweifel *et al*. 2005). The STEC O26, O103, O111, O118, O121, O145 and O157 strains are responsible for the majority of HC and HUS cases worldwide (Tzschoppe *et al.* 2012). The European Food Safety Authority (EFSA) has identified a restricted range of serotypes (i.e. O157, followed by O26, O103, O91, O145 and O111) as public health risks. For these seropathotypes, monitoring should be based on the periodic evaluation of human disease and epidemiological data (Anonymous, 2009).

Domestic and wild animals were reported to be sources of these microorganisms (Bell 2002). Foods of animal origin have been identified as the main vehicles for transmission of *E. coli* O157:H7 and other non-O157 STEC strains to humans. Conversely, foods such as fruits and vegetables are associated with human STEC infections through cross-contamination, *e.g.* inadequate microbial safety precautions during food manipulation and person-to-person transmission. Vegetables may also be considered as a risk for foodborne STEC contamination. Vegetables can become contaminated with STEC through use of manure-based fertilisers, as well as manure-contaminated soil and water (Bell 2002; Franz and Brugen, 2008). Therefore, the

transmission of STEC not only by farm production, but also to children in manure-contaminated environments such as farms, county fairs and farming schools, has serious implications for public health (Gyles 2007). Thus, foods of vegetable origin should be considered as a possible vehicle of STEC transmission (Anonymous 2011). In Turkey, the clinical studies by medical authorities reported that *E. coli* O157:H7 is the most common verotoxigenic *E. coli* serotype. The incidence of STEC cases in Turkey still remains unclear, small outbreaks are not routinely investigated, and studies of this issue are limited (Erdoğan *et al*. 2011). Conversely, other studies conducted in Turkey indicate that foods such as ready to eat salads, meat and meat products, drinking water, fresh fruits, bakery products, and milk and dairy products are contaminated with *E. coli* (Doğan *et al.* 2001; Çıtak *et al*. 2009). The prevalence of the *stx1*, *stx2* and *eae* genes in many *E. coli* serotypes, *e.g.* O157:H7, O111:H2, O119:H6 and O157:NM, in faeces, carcasses of slaughtered cattle and faecally contaminated vegetable samples has been evaluated (Yılmaz *et al.* 2006; Güler *et al.* 2008; Inat and Siriken, 2010).

In this study, we assessed the occurrence of *E. coli* using conventional microbiological methods, identified STEC strains by detecting virulence genes, and assessed the profile of the *E. coli* virulence genes that represent public health and food safety concerns in vegetable samples collected from several district bazaars in Istanbul using real-time PCR.

**RESULTS and DISCUSSION**

This study focused on the assessment and quantification of *E. coli* contamination of vegetables from several district bazaars in Istanbul, and the characterisation of the isolates using real-time PCR. The culture-based results showed that 60.5% (n = 109) of 180 vegetable samples were positive for *E. coli*. According to the results, 93.3% (n =

28) of spinach, 93.3% (n = 28) of lettuce, 86.6% (n=26) of parsley had higher levels, whereas 43.3% (n = 13) of carrot, 33.3% (n = 10) of cucumber and 13.3% (n = 4) of tomato had lower levels of *E. coli* contamination. Of 180 samples, 13 were confirmed to contain STEC (six parsley, three carrots, three lettuces, and one cucumber out of 30 samples of each). Similar studies in Turkey have reported detection of *E. coli* in vegetables and other foods using conventional culture based methods. According to two reports from Ankara, Turkey, 84 salad samples (78.6%) and 60 frozen vegetable samples (33.3%) were positive for *E. coli* (Doğan *et al.* 2001; Çıtak *et al.* 2009). Ayçiçek *et al*., (2006) investigated the bacteriological quality of lettuces, cos lettuce, iceberg lettuce, parsley, dill and carrots. *E. coli* was detected significantly more often in parsley (70%) and dill (40%) samples. Conversely, the lettuce samples showed the lowest rate of *E. coli* contamination (3.3%). These rates are similar for parsley samples, but higher for lettuce samples, than those we report here. However, in the abovementioned study, pathogenic strains of *E. coli* and the relevant virulence genes were not investigated. Many studies conducted in various countries have reported indicated the presence of *E. coli* in vegetables using culture-based methods. In Brazil, Oliveira *et al*., (2012) detected *E. coli* in 53.1% of the leafy vegetable samples analysed, similar to our findings. The rates of *E. coli* contamination in minimally processed salads were only 13.3% in Spain and 3.96% in Portugal (Abadias *et al*. 2008; Santos *et al.* 2012). These results indicate lower levels of *E. coli* compared to our study; this is likely because in these countries salads and vegetables are processed under more hygienic conditions, effective food safety inspections are conducted, and the standard of living is higher than in Turkey.

In our study, the minimum STEC detection level by pre-enrichment for 18 h followed by real-time PCR was 1-10 CFU $ml^{-1}$. Real-time PCR of enrichment cultures of

vegetable samples is superior for detecting pathogenic *E. coli* compared to conventional "cultural and serological" techniques (Liming and Bakhwat, 2004). The absence of non-specific products or primer-dimers was confirmed by performing a melting curve analysis; this showed a single clear melting peak for all real-time PCR assays and no formation of nonspecific products (data not shown). In our study, STEC were detected in 20% (6 out of 30) and 3.3% (1 out of 30) of parsley and cucumber samples, respectively. Additionally STEC was detected in 10% (3 out of 30) of both lettuce and carrot samples. In 13 of 109 (11.9%) STEC-positive samples, 1 sample contained *stx1*, *stx2* and *eae*, 2 samples contained both *stx2* and *eae*, and 10 contained *stx2*. Conversely, our results revealed that 109 *E. coli*-positive samples, 96 (88.1%) were non-STEC, whereas only 13 (11.9%) were identified as STEC serotypes (Table 2). The predominance of STEC type carrying *stx2* sequences in some vegetables, such as carrot, parsley, lettuce, and cucumber, which are consumed raw by the Turkish consumers, represents a serious risk to public health since strains carrying these sequences are frequently associated with serious illness, such as HUS (Griffin and Tauxe 1991). The *wzx* and *wzy* gene sequences were used as diagnostic markers for rapid identification and detection of STEC serogroups (Liua et al. 2007). The results indicated that these were belonging to O157 in parsley, cucumber and carrot, O26 in lettuce, parsley and carrot, and O111 and O113 in parsley only. The remaining four STEC-positive samples could not be serotyped with given specific primers (Table 1). The major STEC serogroups, including O157, O26, O111 and O113, are emerging pathogens associated with HUS and HC in developed countries (Karmali *et al.* 2010). In the present study, these highly virulent seropathotypes were detected in four samples (one lettuce, two parsley, and one carrot). STEC seropathotypes O157 and O157:H7 are frequently isolated from cattle, cattle carcasses and environmental samples from five

abattoirs in İstanbul by multiplex-PCR (Yılmaz *et al.,* 2006). In our study, presence of the *eae* gene in STEC positive samples was associated with O157 serotype, which is in agreement with the report by Sandhu *et al*., (1996). The 10 isolates of serogroups O26 and O111, and O113 did not possess the *eae* gene. Similarly, Fantelli and Stephan (2001) reported that all non-O157 STEC strains isolated from minced meat were negative for *eae* gene. Most outbreaks and sporadic cases of bloody diarrhea and HUS have been attributed to the STEC serotype O157:H7. However, in Europe and recently in the United States, the role of non-O157 STEC strains as causes of HUS, bloody diarrhea, and other gastrointestinal illnesses is being increasingly recognized (Beutin and Martin, 2012). Güler *et al.,* (2008) evaluated the prevalence of genes encoding intimin (*eae*) and Shiga toxins (*stx1* and *stx2*) in 120 *E. coli* isolates from calves by using multiplex PCR assays; no *eae*- or *stx*-positive strains were identified to be O157:H7. In Turkey, pathogenic *E. coli* strains in vegetables originate mainly from insufficient packaging, outdoor selling in bazaars and direct contact with vegetables.

In conclusion, real-time PCR assays used in this study provided valuable information regarding pathogenic *E. coli* contamination of vegetables collected from several public bazaars in the city of Istanbul. These data will facilitate assessment of the risk of contamination by *E. coli*, including STEC serotypes, and characterisation of STEC virulence genes in fresh vegetables consumed in Turkey.

## MATERIALS and METHODS

### *Sample collection*

In this study, a total of 180 vegetable samples (30 spinaches, 30 lettuces, 30 parsleys, 30 carrots, 30 cucumbers and 30 tomatoes) were analysed for the presence of *E. coli* and STEC. The samples were collected randomly from six bazaars from March to

December 2010. The samples were placed into sterile sampling bags using sterile hand gloves, and were immediately transported to the laboratory in a refrigerated container at 4°C until sample preparation and analysis.

***Preparation of samples***

For conventional microbiological analysis, 25 g of each sample was homogenised with 225 ml of buffered peptone water (Oxoid, Wesel, Germany) in a sterile stomacher bag (Interscience Bag System) for 2 min by using a stomacher (AES Laboratoire, Chemunex, France). All the tools -such as forceps and spatula- used for sampling were sterilised by immersion in 96% ethanol and heating over a burner flame. *E. coli* O157:H7 strain NCTC 12900 was obtained from the Food Control Laboratory of Florya-İstanbul under the supervision of the General Directorate of Protection and Control of the Turkish Ministry of Agriculture and Rural Affairs. Homogenised samples in sterilised glass bottles underwent aerobic incubation for 18 h at 37°C. Following incubation, 1 ml of the pre-enriched culture was inoculated onto Tryptone Bile X- Glucuronide (TBX) agar (Oxoid) plates. Carrot, cucumber and tomato samples were incubated for 18 h at 44°C whereas lettuce, parsley and spinach samples for 12 h at 44°C. Blue-colored colonies (indicative of typical *E. coli*) were harvested for DNA extraction by addition of 1 ml of a sterile 0.9% physiological serum isotonic solution (Eczacıbaşı-Baxter A.Ş., İstanbul, Turkey) onto the medium. The solution was spread gently over the agar surface was then carefully re-pipetted and subjected to DNA extraction.

***Extraction of DNA***

DNA was extracted from the *E. coli* isolates on TBX agar plates by following the manufacturer´s protocol (GENE*Spin* DNA Isolation Kit, Eurofins GeneScan GmbH, Freiburg, Germany). Extracted DNA was stored at -20°C.

***PCR primers for STEC and reaction condition***

*E. coli* isolates were subjected to real time PCR detection of STEC using oligonucleotide primers specific for the *stx1*, *stx2* and *eae* virulence genes (Ibekwe *et al.* 2002). The primer sequences are shown in Table 1. *E. coli* O157:H7 strain NCTC 12900 was used as positive control. The primers were obtained from Integrated DNA Technologies BVBA, Leuven-Belgium. SYBR Green master mix was used to increase the sensitivity of the analysis and to prevent false positives due to unintended non-specific amplifications in PCR. First, 12.5 µl of master mix (Eurofins GeneScan), 1 µl of primer mix (each 10 $\mu mol^{-1}$) and 9.5 µl of sterile water were added to each sample and aliquoted into three Eppendorf tubes for the amplification of *stx1*, *stx2* and *eae*. A total of 23 µl of this prepared master mix solution was pipetted into each well following addition of 2 µl of extracted DNA. Each sample was run in duplicate. Sterile water was placed into negative control well in place of DNA, whereas *E. coli* DNA (in two wells) was used as the positive control. FAM fluorescence was used because of the similarity of its excitation and emission spectra to those of SYBR Green (FAM/SYBR® Green 492nm-516nm). Thermal processing parameters were one cycle at 95 °C for 10 min, then 40 cycles at 95 °C for 20 s, 60 °C for 30 s and 72 °C for 30 s, and finally one cycle at 55 °C for 10 min and 95 °C for 30 s. The thermal processing conditions were optimised at the laboratory according to the primers used. To verify the specificity of the reactions using SYBR Green I as a fluorescent dye, melting curve analysis was performed. The analysis was performed using the Agilent Stratagene Mx3000P real-time PCR (Stratagene, Santa Clara, CA, USA). Sensitivity of the real-time PCR assay undergoing $C_t \leq 40$ cycles of amplification was accepted to be positive in accordance with the melting point setting due to the interference of undesirable non-specific

amplifications. The *stx1*, *stx2* and *eae* virulence gene frequencies in the samples were determined based on the method of Ibekwe *et al.* (2002).

***Sensitivity and spiking studies***

To determine the sensitivity of real-time PCR assay for detection of STEC, serial 10-fold dilutions of the *E. coli* O157:H7 strain NCTC 12900 (positive for *sxt1*, *stx2* and *eae*) were inoculated onto tryptic soy agar plates; the density of the initial suspension was then determined by viable counting and expressed as CFU $ml^{-1}$. Subsequently 25 g of vegetable samples (spinach, lettuce, parsley, tomato, carrot and cucumber), which had tested negative for STEC, were inoculated and transferred to buffered peptone water enrichment medium (225 ml). After incubation for 18 h, the real-time PCR was conducted. Two un-inoculated vegetable samples were used as negative controls. DNA from the spiked samples was extracted following the procedure as described above.

***Detection of Serotyping by PCR***

Real-time PCR for serotyping of STEC isolates was performed using specific primers (Table 1). Amplifications were conducted according to the respective references.

**Acknowledgements**

We thank Hürriyet Gazetecilik A.Ş for supporting this food safety study carried out for the public health in İstanbul, Turkey.

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**Table 1**: Primers used in PCR detection of virulence genes and determination of serotype

| Primer | Sequence (5′ - 3′) | $T_m$ ($^0$C) | Target gene | Size of the PCR | Reference |
|---|---|---|---|---|---|
| *stx1* forward | GAC TGC AAA GAC GTA TGT AGA TTC G | 55.6 | *stx1* | 150 | Ibekwe *et al.* (2002) |
| *stx1* reverse | ATC TAT CCC TCT GAC ATC AAC TGC | 56.2 | | | |
| *stx2* forward | ATT AAC CAC ACC CCA CCG | 55 | *stx2* | 200 | |
| *stx2* reverse | GTC ATG GAA ACC GTT GTC AC | 54.2 | | | |
| *eae* forward | GTA AGT TAC ACT ATA AAA GCA CCG TCG | 55.6 | *eae* | 106 | |

| | | | | | |
|---|---|---|---|---|---|
| *eae* reverse | TCT GTG TGG ATG GTA ATA AAT TTT TG | 52.3 | | | |
| wzxO26 F | CAG AAT GGT TAT GCT ACT GT | 60 | *wzx* | 423 | Posse´ *et al.* 2007 |
| wzxO26 R | CTT ACA TTT GTT TTC GGC ATC | 54 | | | |
| wzxO103 F | TTGGAGCGTTAACTGGACCT | 57 | *wzx* | 321 | |
| wzxO103 R | GCTCCCGAGCACGTATAAG | 57 | | | |
| wzxO111 F | TAG AGA AAT TAT CAA GTT AGT TCC | 62 | *wzx* | 406 | |
| wzxO111 R | ATA GTT ATG AAC ATC TTG TTT AGC | 62 | | | |
| wzxO145 F | CCA TCA ACA GAT TTA GGA GTG | 59 | *wzx* | 609 | |
| wzxO145 R | TTT CTA CCG CGA ATC TAT C | 59 | | | |
| wzxO157 F | CGG ACA TCC ATG TGA TAT GG | 60 | *wzx* | 259 | |
| wzxO157 R | TTG CCT ATG TAC AGC TAA TCC | 60 | | | |
| wzxO45-F | CCG GGT TTC GAT TTG TGA AGG TTG | 59 | *wzx* | 527 | DebRoy *et al.* 2005 |
| wzxO45-R | CAC AAC AGC CAC TAC TAG GCA GAA | 59 | | | |
| wzxO113-F | GGG TTA GAT GGA GCG CTA TTG AGA | 60 | *wzx* | 771 | DebRoy *et al.* 2004 |
| wzxO113-R | AGG TCA CCC TCT GAA TTA TGG CAG | 60 | | | |

**Table 2**. Occurrence of STEC virulent genes in different vegetable samples and obtained serotypes.

| **Sample** | **No. of samples** | **Growth of *E. coli*** | **STEC** | ***stx*2** | **both *stx*2 & *eae*** | **all the 3 genes** | **Serotype** |
|---|---|---|---|---|---|---|---|
| **Spinach** | 30 | 28 (93.3%) | n.d. | | | | n.d. |
| **Lettuce** | 30 | 28 (93.3%) | 3 (10%) | 1 | | | O26 |
| | | | | | 1 | | O157 |
| | | | | 1 | | | n.d. |
| **Parsley** | 30 | 26 (86.6%) | 6 (20%) | 1 | | | O26 |
| | | | | 1 | | | O111 |
| | | | | 1 | | | O113 |
| | | | | | 1 | | O157 |
| | | | | 2 | | | n.d. |
| **Carrot** | 30 | 13 (43.3%) | 3 (10%) | 1 | | | O26 |
| | | | | 1 | | | n.d. |
| | | | | | | 1 | O157 |
| **Cucumber** | 30 | 10 (33.3%) | 1 (3.3%) | 1 | | | 026 |
| **Tomato** | 30 | 4 (13.3%) | n.d. | | | | n.d. |
| **Total** | 180 | 109 (60.5%) | 13 (11.9%) | 10 (9.2%) | 2 (1.8%) | 1 (0.9%) | |

The numbers in parenthesis represent percent values.

n.d.: not detected