VIRULENCE GENE PROFILES OF SHIGA-TOXIN PRODUCING *ESCHERICHIA COLI* ISOLATES FROM RETAIL RAW MEAT IN IRAN

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Summary

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Shiga toxin-producing *Escherichia coli* (STEC) is recognised as toxin producing group of *E. coli* and one of the most significant foodborne pathogens worldwide. The aim of this study was to detect STEC and determine virulence gene profiles of these pathogens in different kinds of meat and products in Iran. For this reason a total of 182 samples of minced beef, mutton, chicken meat, chicken feet and mechanically separated chicken meat were collected from retail markets for detection of STEC by PCR method. Of the 72 *E. coli* isolated from examined samples, 29 of them were STEC. The highest presence of STEC was detected in minced beef (23.5%) followed by chicken feet (15%), mutton (13.3%), mechanically separated chicken meat (12.5%) and chicken meat (5.5%) respectively. In addition the results of PCR assay indicated that 21 (72.4%) and 4 (13.7%) of isolates carried *stx2* and *eaeA* genes respectively. However, according to the results *stx2* was the most prevalent gene detected in all kinds of examined samples. Our findings showed that evaluation of the prevalence and virulence factors of this pathogen seems necessary considering the increasing importance of STEC as one of the most significant foodborne pathogens.

Key words: beef, chicken, mutton, *eaeA*, Shiga toxin-producing *Escherichia coli*, *stx1*, *stx2*

INTRODUCTION

Shiga toxin-producing *Escherichia coli* (STEC) is recognised as toxin producing group of *E. coli* and one of the most significant foodborne pathogens worldwide (Farrokh *et al.*, 2013). Wide range of human clinical symptoms comprising diarrhoea, haemorrhagic colitis (HC) and haemolytic uremic syndrome (HUS) have been attributed to both non-O157 STEC and O157 isolates (Paton & Paton, 1998;

Wang *et al.*, 2013). Among several serogroups of STEC, O157:H7 is the most abundant serogroup related to food or foodborne diseases (Farrokh *et al.*, 2013; Smith *et al.*, 2014). Recent FoodNet data suggest that non-O157 STEC infections have started to gain predominance over O157 cases in the United States (Thorpe, 2004; Wang *et al.*, 2013). Domestic ruminants and especially cattle are recognised

as predominant reservoir of STEC (Chinen *et al.*, 2003; Meichtri *et al.*, 2004), although this pathogen can be transmitted to humans through consumption of several foods of non-bovine origin including unpasteurised juices, salami, cheese, raw (unpasteurised) milk and water (Throp, 2004; Smith *et al.*, 2014). There are several reports associated with outbreaks of STEC around the world. In a massive outbreak in China, 195 hospitalised HUS patients and 177 deaths have been reported (Wang *et al.*, 2011; Xiong *et al.*, 2012). In another outbreak caused by non-O157 STEC, infected cases including 43 deaths have been recently reported in the European Union (ECDC, 2011). Furthermore, although the pathogenicity mechanisms of STEC are not yet fully understood, Shiga toxin genes 1 and 2 (*stx1* and *stx2*) are the main factors for virulence properties in STEC pathogenesis and development of clinical symptoms (Kawano *et al.*, 2012; Bai *et al.*, 2015). Moreover, previous literature reports indicated that other virulence factors may play significant roles in the pathogenicity of this pathogen such as intimin which is encoded by the chromosomal gene *eaeA* and is responsible for intimate attachment of STEC to intestinal epithelial cells (Kaper *et al.*, 1998; Bastos *et al.*, 2006). Therefore, due to the importance of STEC in

human public health, the purpose of this study was to detect STEC and determine virulence gene profiles of these pathogen in different kinds of meat and products in Iran.

MATERIALS AND METHODS

Sample collection

A total of 182 samples of minced beef (68), mutton (30), chicken meat (36), chicken feet (40) and mechanically separated chicken meat (8) were collected from retail markets in Alborz Province between September 2014 and February 2015. All samples were transferred under cold conditions to the laboratory for STEC identification.

Sample preparation

To count and detect *E. coli*, 25 g of each sample product was aseptically weighed and added to a sterile bag containing 225 mL of sterile 0.01% (w/v) buffered peptone water (10^{-1} dilution) (Merck, Germany). Samples were homogenized for 1 min in a BagMixer® 400 lab blender (Interscience, France). For detection of STEC, 0.1 mL of the dilution was transferred onto MacConkey agar and incubated at 37 °C for 18 to 24 h. Then, tentative *E. coli* colonies were identified by biochemical tests. Indol and methyl red

Table 1. Primers used for PCR and DNA sequencing (Franck *et al.*, 1998)

Virulence factor	Primer sequence 5–3′	Position in open reading frame	Product size
<i>stx1</i>	TTCGCTCTGCAATAGGTA	125–142 of A subunit	555
	TTCCCCAGTTCAATGTAAGAT	659–679 of A subunit	
<i>stx2</i>	GTGCCTGTTACTGGTTTTCTTC	30–53 of A subunit	118
	AGGGGTCGATATCTCTGTCC	128–147 of A subunit	
<i>eaeA</i>	ATATCCGTTTAAATGGCTATCT	992–1013 of <i>eaeA</i>	425
	AATCTTCTGCGTACTGTGTTC	1395–1416 of <i>eaeA</i>	

positive and Voges-Preskauer and Simmons citrate negative colonies were confirmed as *E. coli* and subcultured in nutrient broth at 37 °C for 18 to 24 h.

DNA extraction

Overnight cultures in 2 mL nutrient broth were centrifuged for 5 min at 5,000 rpm. The bacterial pellet was re-suspended in 200 µL of distilled water and boiled for 10 min. Tubes were centrifuged again, and the supernatant was used as template DNA (Pourtaghi *et al.*, 2013).

STEC detection

PCR was performed on the samples to detect the presence of the *stx1*, *stx2*, and *eae* genes. Three sets of primers previously described (Frank *et al.*, 1998) were used in the present study. The primer sets and related genes that encode virulence genes are described in Table 1. The primer sets were synthesised by Cinna-Gene, Iran. Amplification reactions were performed in a total volume of 25 µL containing 2.5 µL of 10× PCR buffer, 0.5 mM MgCl₂, 250 µM dNTP, 1 µM of each primer and 0.5 U Taq DNA polymerase. The condition for amplification consisted of 94 °C for 5 min for initial denaturation and 35 cycles of 94 °C for 1 min (denaturation), 50 °C for 1 min and 72 °C for 1

min 30 s (extension), followed by 72 °C for 10 min (final extension). Amplified products were separated and visualised by standard gel electrophoresis using 10 µL of the final mixture in 1% agarose gel in TBE buffer (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA) containing 0.5 mg ethidium bromide. To determine molecular weight, 100–1,000 bp DNA ladder (Fermentas) was used.

RESULTS

Of the 72 *E. coli* isolated from examined samples, 29 of them were STEC. Table 2 shows the prevalence of STEC isolated from examined samples. The highest prevalence of STEC was detected in minced beef (23.5%) followed by chicken feet (15%), mutton (13.3%), mechanically separated chicken meat (12.5%) and chicken meat (5.5%) respectively. In addition the results of PCR assay indicated that 21 (72.4%) and 4 (13.7%) of isolates carried *stx2* and *eaeA* genes respectively. Although *stx2* was detected from all kinds of examined samples, *eaeA* gene was only identified from chicken feet. Furthermore, *stx1* was detected only in combination with *stx2* and *eaeA* from 2 (6.9%) of examined samples which both of them were

Table 2. Prevalence of STEC isolated from examined samples

Samples	Number of samples	Number (%) of positive samples
Minced beef	68	16 (23.5)
Mutton	30	4 (13.3)
Chicken meat	36	2 (5.5)
Chicken feet	40	6 (15.0)
Mechanically separated chicken meat	8	1 (12.5)
Total	182	29 (15.9)

Table 3. Prevalence of *stx1*, *stx2* and *eaeA* genes in *E. coli* isolated from retail raw meat

Virulence gene(s)	No. of strains with indicated virulence gene(s)					Total (%)
	Minced beef	Mutton	Chicken meat	Chicken feet	Mechanically separated chicken meat	
<i>stx1</i>	0	0	0	0	0	0 (0)
<i>stx2</i>	12	4	2	2	1	21(72.4)
<i>eaeA</i>	0	0	0	4	0	4 (13.7)
<i>stx2</i> & <i>eaeA</i>	2	0	0	0	0	2 (6.9)
<i>stx1</i> , <i>stx2</i> & <i>eaeA</i>	2	0	0	0	0	2 (6.9)

Fig. 1. Agarose (1%) gel electrophoresis of STEC PCR products of virulence factors genes.

Lane 1: positive isolate for both *stx2* and *eaeA* genes (118 and 425 bp), lanes 2 and 3: *eaeA* positive isolates (425 bp), lane 4: positive control, lanes 5–7: negative control and negative isolates; lane M: DNA molecular weight marker (100–1,000 bp).

from minced beef. Moreover, similar result was observed for the combination of *stx2* and *eaeA* genes. However, according to the results *stx2* was the most prevalent gene detected from all kinds of examined samples. The prevalence of these detected genes is shown in Table 3. Fig 1. shows the virulence genes of some of isolates.

DISCUSSION

In recent decades STEC is acknowledged as an emerging foodborne pathogen leading to several outbreaks or sporadic cases of HC and HUS (Karmali *et al.*, 2010). The results of the present study demonstrated that meat and meat products possess a high potential for causing human infection. Our findings indicated that 23.5% of minced beef samples were contaminated with STEC which was lower than that reported in previous investiga-

tions in Iran (29.7%) (Momtaz *et al.*, 2013) and Argentina (52.5%) (Brusa *et al.*, 2013) but higher than the result found in Switzerland (1.75%) and New Zealand (12%) (Brooks *et al.*, 2001; Fantelli & Stephan, 2001). Considering the rising public health concern about non-O157 STEC, since 2012, Department of Agriculture (USDA)'s Food Safety and Inspection Service, expanded the zero tolerance policy for *E. coli* O157:H7 to include the top six non-O157 STEC serogroups in raw beef products (Wang *et al.*, 2013). Furthermore, although the contamination rate of mutton in this study (13.3%) was lower than the results reported in Iran (35.4%; Momtaz *et al.*, 2013) and New Zealand (17%; Brooks *et al.*, 2001), it was higher than the rate observed in previous investigation in Italy (7.1%; Franco *et al.*, 2009). In addition, according to the results chicken feet showed higher contamination than chicken meat, whereas previous investigations represented lower rate of contamination with STEC in chicken meat and products (Xia *et al.*, 2010; Bai *et al.*, 2015). Isolation of STEC from chicken meat and products represented that in addition to ruminant's meat as the major source of STEC, poultry meat may be another reservoir of contamination with this pathogen (Dipineto *et al.*, 2006). These observable differences in prevalence can be due to diversity in sampling methods, geographic area, types of samples, methods and techniques (Momtaz *et al.*, 2013).

Shiga-toxins are the most significant virulence factor in pathogenesis of STEC (Scheut *et al.*, 2012). *Stx1* and *stx2* are known as the initial virulence factors of STEC. In contrast to STEC that produce *stx1* only, STEC that produce *stx2* alone or both *stx1* and *stx2* are more likely to be

associated with HUS (Johnson *et al.*, 2006). So, *stx2*-producing strains are more relevant to clinical disorders including HUS than *stx1* (Paton & Paton, 2002; Bai *et al.*, 2015). In the present study most of the isolates possessed *stx2* which is in accordance with the results reported in Argentina (Blanco *et al.*, 2004), China (Zheng *et al.*, 2005; Koitabashi *et al.*, 2008), Japan (Kawano *et al.*, 2012) and Denmark (Nielsen *et al.*, 2002). Lower prevalence rates were reported in Brazil (1.35%; Martins *et al.*, 2011) and India (11%; Khan *et al.*, 2002). Furthermore, the results of this study showed that in addition to *stx2* as the predominant gene, *eaeA* gene was the second most detected gene from STEC isolates. STEC that contain the "locus of enterocyte effacement" pathogenicity island, or "LEE region" colonise the lower gastrointestinal tract. The LEE region genes (*eaeA*) stimulate intestinal cells to build surface proteins permitting *E. coli* to adhere intimately to gut epithelium (Thorpe, 2004). Non-O157 STEC containing LEE are more frequently associated with HUS and outbreaks than are LEE-negative strains (Johnson *et al.*, 2006). Our findings confirmed that although *stx2* was found in all kinds of examined samples, *eaeA* gene has been isolated only from chicken feet. Moreover, in contrast to our results, prevalence of virulence genes including *stx1*, *stx2* and *eaeA* in chicken meat samples was much higher in previous investigation in Iran (Momtaz & Yamshidi, 2013). Therefore, according to the literature, the presence of these virulence genes indicated that consumption of raw or undercooked meat and meat products can cause diseases such as HUS and HC in humans (Momtaz & Yamshidi, 2013). Similarly to the results of previous studies (Blanco *et al.*, 2004) our findings indi-

cated that the evaluation of the prevalence and virulence factors of this pathogen seems necessary considering the increasing importance of STEC as one of the most significant foodborne pathogens.

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