Detection and Frequency of Stx2 Gene in Escherichia coli O157 and O157:H7 Strains Isolated from Sheep Carcasses in Shiraz-Iran

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Abstract: Enterohaemorrhagic Escherichia coli constitute a subset of serotypes (E. coli O157 and some other serogroups) of Shiga toxin-producing E. coli firmly associated with severe human illnesses like bloody diarrhea and haemolytic uraemic syndrome. Escherichia coli O157:H7 is a zoonotic pathogen. They rarely cause disease in animals, live in the intestines of healthy sheep and ruminants are recognized as their main natural reservoir, so they can contaminate meat during slaughtering practices. The purpose of this study was epidemiological survey on the occurrence of E. coli O157:H7 in healthy sheep in Shiraz-Iran. Polymerase Chain Reaction (PCR) assay was developed to detect the Stx, gene the only bacterial factor that has been associated with more severe disease. During a period of 7 months (December 2005 to June 2006), 153 slaughtered sheep at Shiraz slaughterhouse, were randomly selected and examined for surface carriage of E. coli O157:H7 by conventional plating and Stx gene detection by PCR technique. E. coli O157:H7 was found in 6(3.92%) of 153 sheep. The bacteria were isolated from 5(3.3%) of 114 and 1(2.63%) of 38 sheep two or under two and more than 2 years old, respectively (p = 0.5). The contamination rate might vary depending on season, age and infection time. The higher frequency for younger animals may be due to differences in the composition of the gastrointestinal flora resulting from differences in diet. This is the first report of the presence of E. coli O157:H7 in sheep from Iran.

Key words: Escherichia coli O157:H7, sheep carcasses, SMAC, PCR

INTRODUCTION

Escherichia coli O157:H7 is the causative agent of human diseases, including mild diarrhea, hemorrhagic colitis and the diarrhea-associated form of the Hemolytic-Uremic Syndrome (HUS) (Griffin and Tauxe, 1991). This bacterium was first recognized as a human pathogen in 1982 (Riley et al., 1983). Most reported outbreaks of E. coli O157:H7 infection has been associated with contaminated cattle products, such as undercooked beef (Heuvelink et al., 1998) or raw milk (Vernozy-Rozand et al., 2005). Since healthy domestic animals, in particular, ruminants like cattle, sheep and goats, can harbor verotoxin producing E. coli VTEC in their feces, they are regarded as natural reservoirs of these pathogens (Beutin et al., 1993).

The major virulence characteristics of Shiga toxin (Stx)-producing E. coli (STEC) strains are production of Shiga toxins (Garcia-Aljarro et al., 2004). The Stx family contains two major, immunologically non-cross-reactive groups called Stx1 and Stx2 (Nataro and Kaper, 1998).

Epidemiological data suggest that Stx2 is more important than Stx1 in the development of HUS (Pickering et al., 1994). Contamination of meat with STEC from bovine feces occurs during slaughter or meat processing and is the major route by which these pathogens enter the food chain (Armstrong et al., 1996).

There are no common biochemical characteristics being associated with the great majority of EHEC serotypes. However, there are some biochemical characteristics of E. coli O157:H7 that have been exploited in the isolation and identification of this serotype. The agar medium most commonly used for the isolation of E. coli O157:H7 is Sorbitol McConkey (SMAC) agar supplemented with cefixime 0.05 mg L⁻¹ and Potassium Tellurate 2.5 mg L⁻¹ (Sanderson et al., 1995). An important characteristic is that E. coli O157:H7 strains do not ferment D-sorbitol rapidly, in contrast to about 75 to 94% of other E. coli strains (March and Ratnam, 1986).

A very low infectious dose for Enterohaemorrhagic Escherichia coli (EHEC) infection has been estimated from outbreak investigations. This number, on the order...
of 100 to 200 organisms for infection (Nataro and Kaper, 1998), so a sensitive and specific methods are need.

Several techniques such as immunoassays, Vero cell assay and PCR have been used to detect verotoxins or their genes (Philips, 1999). Most of these methods require an enrichment step in order to increase their respective threshold of detection (Lionberg et al., 2003). Several types of enrichment broths are currently used for STEC before their detection by screening methods (Cui et al., 2003; McKee et al., 2003). Furthermore, some researchers incorporate selective agents (such as various antibiotics and bile salts) in the broths (Catarame et al., 2003; Foster et al., 2003). Amongst these, PCR has been widely used and a number of studies have targeted Stx genes and one or more E. coli O157:H7-specific genetic markers (Yilmaz et al., 2006).

As a contribution to the understanding of the epidemiology of human EHEC infections, the present study describes the isolation and identification of E. coli O157:H7 strain from sheep in Shiraz-Iran. The samples were examined for the presence of O157 VTEC by performing both conventional plating and then PCR. To determine the isolates as potential human pathogens, they were tested for just presence of main virulence-associated gene (Stx2).

Hence, this study was performed in order to describe the identification of E. coli O157:H7 from slaughtered sheep at Shiraz slaughterhouse by CT-SMAC and then with detection Stx2 gene by PCR.

MATERIALS AND METHODS

Sampling: The size of the sampling has been fixed to 153 sheep, which is sample size collection at the three slaughterhouses of the Shiraz Iran from December 2005 to June 2006 at 7 months period. Systematic sampling was performed and 1:5 ratios were used for selecting in proportions representative sheep at different age.

After de-hiding and evisceration, sampling method was used as described by Bolton et al. (2001). The swab were rubbed to an area of 25 cm² in three different high risk fecal contamination areas (neck, shoulder and abdomen) (Fig. 1) and aseptically placed into 15 mL modified tryptcase-soy broth (Merck) supplemented with cefoxime (0.15 mg L⁻¹) and potassium tellurite (5 mg L⁻¹) (Sigma) (mTTSB). Samples, kept cold with melting ice, when transported to the laboratory.

Preenrichment: Immediately upon arrival, microbiological analysis was started as described bellow. The samples were incubated at 37°C over night for enrichment. The samples grown in mTTSB were processed in two steps:

**Conventional plating:** The samples were streaked on CT-SMAC and incubated at 37°C over night. After incubation, the plates were checked for the presence of sorbitol negative (pale color) colonies. These colonies were picked and further identification was performed using indol, rhamnose, urease activity, motility and CT-SMAC again and putative E. coli O157 was identified as sorbitol non-fermenting colonies. The pale colonies were re-plated on CT-SMAC again to yield separated single colonies for purification (Willshaw et al., 2001).

For confirmation, the single pale colony was used for serotyping. O grouping was carried out by slide agglutination of living bacteria with antisera specific for the major Enterohaemorrhagic E. coli groups O157 (Difco antisera). The O157 positive serotypes were chosen for PCR assay as describe below (Rogeri et al., 2001; Willshaw et al., 2001).

**Serogrouping:** Specific E. coli O157 antisera (Difco-2970-47) and also H7 flagella was used to determine the serotype of bacteria by agglutination test according to manufacturer. The O157 positive serotypes were chosen for PCR assay as describe below (Rogeri et al., 2001; Willshaw et al., 2001).

**PCR**

**Bacterial DNA extraction:** We used modified in-house enrichment methods for DNA extraction. 1.3 mL of overnight mTTSB enrichment culture was transferred to a 1.5 mL clean microtube and the tube was centrifuged at 14000 xg for 5 min. The supernatant was removed and the resultant pellet was resuspended in 150 μL Triton X-100 lysis buffer (100 mM NaCl, 10 mM Tris-HCl (pH 8.3), 1 mM EDTA (pH9), 1% Triton X-100 (Sigma Chemical Co.). The sample then was boiled for 10 min, cooled and centrifuged at 14000 xg for 1 min. A 50 μL aliquot of the supernatant
was prepared and preserved as the PCR template (Holland et al., 2000; Gourmelon et al., 2006).

**PCR and nested PCR-based detection of Stx2 genes:** To evaluate the presence of Stx2 gene, a pair of primers for Stx2 were used. Oligonucleotides primers used for PCR and Nested PCR were synthesized by Roche, Germany, based on published data (Gannon et al., 1999; Meyer et al., 1992). Primer for the amplification of Stx2 sequences were forward, and II-R, that resulting in a PCR product of 779 bp, while for the nested system II-F and II-R yielded a fragment of 372 bp as shown in Table 1.

PCRs were performed with some modifications to the protocol described by Holland et al. (2000). Twenty-five microliter reactions mixture contained: 0.2 μM of each specific oligonucleotide primers for Stx2, 0.2 μM of each deoxyribonucleotide triphosphates, 1X PCR buffer (10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.001% gelatin), 1.5 mM MgCl2, 2.5 U of thermostable DNA polymerase (Taq polymerase Roche Molecular system) and 2 μL DNA template for first PCR and 1 μL MgCl2 and 0.3 μM of each Stx2, primers for the second, Nested PCR with the primer pairs II-F/II-R, The reactions were carried out with a Techne ThermoCycler (England) and cycling conditions with some modification as follows: 94°C for 2 min, 30 cycles of 94°C for 30 sec, 57°C for 45 sec and 72°C for 30 sec and a final extension at 72°C for 10 min. After each PCR assay, 10 μL of the amplification product were analyzed on 1% agarose (Merck) gels containing 0.4 μg mL⁻¹ ethidium bromide, visualized under UV illumination, saved and photographed. Each agarose gel electrophoresis run included DNA molecular size standards (100 bp Ladder) (Holland et al., 2000; Piccozzi et al., 2005).

**Sensitivity and specificity of the PCR:** To estimate the assay sensitivity, 10-fold serial dilutions of certain *E. coli* (serotype O157:H7 AC425) in 0.1% peptone-water were prepared and the suspensions containing 10⁸ to 10⁰ colony forming unit (CFU) mL⁻¹ were amplified without prior enrichment and also with overnight enrichment. The PCR procedure was done as described above and the PCR products were run on 1% agarose gel. To determine the specificity of the applied PCR several enteric bacterial strains including VT-producing *E. coli* of various serotypes such as O1, O2, O6 and O55, Salmonella sp., Klebsiella sp. and Listeria sp. were used.

**Data analysis:** Data was analyzed by Chi-square, Fisher’s exact and McNemar test.

**Table 1:** Primers used in PCR and Nested PCR for amplification of STX2

<table>
<thead>
<tr>
<th>Primers</th>
<th>Oligonucleotide sequence (5’-3’)</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>STx-F1</td>
<td>CCT TGA CAA CCA GCA GCA GTT</td>
<td>779</td>
</tr>
<tr>
<td>STx-R1</td>
<td>GGT CTC GCT CTT TTT GTC ACT GT</td>
<td>372</td>
</tr>
<tr>
<td>STx-R2</td>
<td>AGC TGT ATT ACT TTC CCA TAA</td>
<td></td>
</tr>
</tbody>
</table>

**RESULTS**

*E. coli* O157:H7 was isolated from 6 (3.92%) samples by direct PCR. Eleven sorbitol negative colonies on CT-SMAC agar were identified. According to Table 2, nine motile, 5 rhamnose positive, 7 urease positive and 8 indol positive isolates were identified. Seven (5.57%) samples reacted with the O antisera. Four (2.61%) of 153 samples that were detected from carcasses surface confirmed by PCR (Table 3). Figure 2 shows positive PCR for some samples. Positive PCR was confirmed by Nested PCR (Fig. 3).

Some information about age is shown in Table 4. *E. coli* O157 strains were isolated from 5 (3.34) of 114 and 1 (2.63%) of 38 sheep two or under two and more than two years old, respectively (p = 0.5) (For statistical analysis, we mixed the ages more than two years old because of a few number of samples). There is no differences in sheep according to ages (p = 0.65).

**Comparison the detection methods:** The direct PCR technique proved to be significantly more sensitive for detection of *E. coli* O157 than the conventional plating methods and comparison with PCR technique after enrichment and CT-SMAC harvested (p=0.026) (Table 3).

**Table 2:** Cultural and biochemical characteristic of sorbitol negative bacteria isolated from sheep carcasses, Shiraz-lam (2005-2006)

<table>
<thead>
<tr>
<th>Abattoir No.</th>
<th>SN</th>
<th>MI</th>
<th>RP</th>
<th>UP</th>
<th>IP</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>3</td>
<td>2</td>
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</table>

**Table 3:** Isolation rate of *E. coli* O157:H7 from cattle and sheep at Shiraz slaughterhouse according to different methods (2005-2006)

<table>
<thead>
<tr>
<th>No. of animal</th>
<th>Direct PCR (%)</th>
<th>CT-SMAC (%)</th>
<th>O157 antisera (%)</th>
<th>PCR + source of isolate (%) +</th>
<th>Positive (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>153</td>
<td>6(3.92)%</td>
<td>11(7.18)%</td>
<td>7(5.57)%</td>
<td>4(2.62)%</td>
<td></td>
</tr>
</tbody>
</table>

Different superscripts letter(s) in each columns denote significant differences (p<0.026)
Fig. 3: Stx2 of *E. coli* O157. Products of Nested PCR on some *E. coli* O157:H7 isolates from sheep. Lane 1: 1000 bp ladder marker, Lane 2: Negative control, Lane 3-5: 372 bp positive samples.

Fig. 4: Stx2 of *E. coli* O157. PCR products of a 10-fold serial dilution of *E. coli* O157:H7 (without enrichment). Lane 1: 1000 bp ladder marker, Lane 2: Negative control, Lane 3-11: Bacterial suspensions containing 10^4 to 10^7 cfu mL^{-1}, respectively.

Fig. 5: Stx2 of *E. coli* O157. PCR products of a 10-fold serial dilution of *E. coli* O157:H7 (after overnight enrichment). Lane 1: 100 bp ladder marker, Lane 2: Negative control, Lane 3-8: Bacterial suspensions containing 10^7 to 10^9 cfu mL^{-1}, respectively.

Stx2 PCR and 372 bp for the Stx2 Nested PCR systems. No amplicons were obtained from strains that did not contain the Stx2 gene (Fig. 4).

**DISCUSSION**

Although many types of food have been associated with VTEC infections, undercooked ground meat is probably the main vehicle of transmission to humans (CDC, 1995). It is important to know the contamination during slaughter since the majority of food-borne *E. coli* O157:H7 infections in humans have occurred after consuming contaminated meat products (Chapman *et al.*, 1993).

This study was designed to identify and to confirm the contamination of *E. coli* O157:H7 on slaughtered sheep carcasses at Shiraz slaughterhouse-Iran, to establish the sensitivity and specificity of the Stx2 primers in the PCR and Nested PCR protocol and to compare PCR and Nested PCR with conventional method. In this study, we have been able to easily detect as little as 10^2 *E. coli* O157:H7 per mL of peptone water.

In present study, we wanted to detect *E. coli* O157:H7 in the presence of other *E. coli* strains. The strain of *E. coli* associated with Hemorrhagic Colitis (HC) and HUS has two properties that made it easy to be differentiated from other *E. coli* strains. It does not ferment D-sorbitol within 2 days, whereas about 95% of other *E. coli* does it. It had H7 antigen, but over 90% of other *E. coli* strains are either non motile or have a different H antigen (Farmer and Davis, 1985).

Sheep the second most commonly reared species of ruminant food animals appears to have a role similar to that of cattle as a natural reservoir of O157 VTEC (Heuvelink *et al.*, 1998). In this study, the prevalence of sheep carcasses contamination with *E. coli* O157 was 6 (3.92%) of 153 sample collection. Results in surveys
carried out by Chapman and Ashton (2003) in Europe have generally shown a very low prevalence of the organism on sheep carcasses (Bonardi et al., 2001), where as E. coli O157 has been found in 10.7% of sheep carcasses, a far higher prevalence than reported in this study (Chapman et al., 2001; Chapman and Ashton, 2003).

Kudva et al. (1996) studied 35 free-ranging healthy ewes of a single flock in Idaho and reported that the incidence of fecal shedding varied from 31% of sheep in June to none in November. In the United Kingdom, O157 EHEC strains were found in the feces of 18 (2.6%) from 700 sheep sampled at a slaughterhouse. Heuvelink et al. (1998) reported isolation of O157 EHEC strains from about 4.9% of Dutch sheep. The ability to compare published prevalence data is limited because of the use of a large variety of screening methods.

The overall E. coli O157:H7 prevalence in 153 investigated sheep is low as compared to what is found in other countries (Bonardi et al., 1999; Elder et al., 2000). The low prevalence in domestic animals is reflected in the low incidence of E. coli O157:H7 infections in that area (Johnsen et al., 2001).

E. coli O157 strains were isolated from 5(3.34%) of 115 sheep two or below two years old and 1(2.63%) of 38 sheep over two years old (Table 3). There is no significant differences in prevalence according to age (p = 0.60).

Fecal and hide prevalence of E. coli O157:H7 has been reported to be significantly correlated with carcass contamination (Gun et al., 2003).

It has also been reported that carcasses can be contaminated from the conveying equipment during process (Gill and Jones, 2000). The findings in the studies mentioned above have indicated that the main source of contamination was faecal pollution before and during slaughterhouse processing. These results indicate that contamination from the faeces on the hides or from intestinal contents to carcasses might be occurring during slaughterhouse processing (Gun et al., 2003).

Isolation rate of E. coli O157:H7 in present study was similar or higher than that of the reported studies conducted in European countries. The reason for this discrepancy might be due to the differences in the examined geographical regions, season, high number of microorganisms in the sheep intestine and environment or different methodologies used for the isolation of E. coli O157 (Aslantas et al., 2006). The surface swabbing method, which was used in this study, was also used by others and is recommended as an alternative method since it is cost-effective and nondestructive (Park et al., 1994; Gun et al., 2003).

It is difficult to directly compare results from different studies due to varying sampling and testing regimes. In the present study, we used the PCR technique for identification of E. coli O157:H7, a method which is regarded as one of the most sensitive identification methods for E. coli O157:H7 (Johnsen et al., 2001). The results were confirmed by Nested PCR and then compare with conventional methods.

For detection of lower numbers of the pathogen, prior enrichment will be necessary. In this study, we could easily detect the Stx genes in as little as 10^3 cfu mL^-1 without prior enrichment and 10^2 cfu mL^-1 after over night enrichment of bacterial suspension. This result is in consistent with Pollard et al. (1990). Results of positive controls of the above described experiment confirmed this detection limit.

The initial assessment of the sensitivity of the PCR for detecting E. coli O157 in inoculated beef samples suggested that PCR may be more sensitive than culture for detecting the organism after enrichment and IMS. Attempts have been made to overcome this delay by performing PCR on directly on extracts of food, but this has been less sensitive than PCR performed on broth enrichment cultures (Gannon et al., 1999). Broth enrichment probably increases sensitivity of the PCR both by dilution of inhibitors and by increasing the numbers of the target organism (Chapman et al., 2001).

In conclusion, this investigation, that was the first epidemiological survey on the occurrence of E. coli O157:H7 in healthy sheep in Iran, confirms that in Iran, as well as the other countries, sheep represent the second reservoir of non-sorbitol fermentive EHEC O157 and the prevalence of the bacterium appears to be similar to those to the other parts of Asia (Yilmaz et al., 2006). Cattle isolation rate in Iran 9.74%, data was not published). Despite the increasing significance of EHEC O157:H7 strains in the etiology of the disease in Iran, the epidemiology of infections caused by these strains is poorly understood.

As for other zoonotic agents, having animals and raw products that are free from EHEC is not possible in practice. However, their occurrence can be minimized by applying high standards of hygiene in all steps of the food production chain (Caprioli et al., 2005).

Further studies are now needed to identify the all VTEC and also vehicles of infection most relevant to our epidemiologic situation and the most appropriate measures to prevent the spread of this life-threatening food borne disease.

Although the PCR technique used in this study took less than 3 h to perform, it was preceded by 24 h of enrichment culture. Attempts have been made to overcome this delay by performing PCR on direct extracts of food, but this has been less sensitive than PCR performed on broth enrichment cultures (Gannon et al., 1999).
Broth enrichment probably increases sensitivity of the PCR both by dilution of inhibitors and by increasing the numbers of the target organism (Chapman et al., 2001). It is suggested that, Iranian sheep also are an important reservoir of E. coli O157 and potentially can cause serious disease in humans. The use of sensitive detection techniques is essential for detection of this pathogen in fecal samples. From the public health point of view, the high percentage of strains harboring the Stx genes with a higher level of expression raises the risk of human infections. PCR has appeared to be a highly sensitive method for distinguishing between apparently unrelated E. coli O157 strains. By analyzing the relationship between these pathogens and the farm environment, we hope to eventually reduce the risk of O157 VTEC-positive animals going to slaughter and in turn, the risk of O157 VTEC infections in humans. Therefore, further studies need to be designed to reduce the occurrence of E. coli O157:H7 in cattle and sheep and the occurrence rate of food borne diseases implicated with these animals caused by E. coli O157:H7 in Iran.

These results will have substantial implications for research aimed at understanding the biology of this important food-borne organism. It is possible to envisage treatments aimed at eradicating the bacteria from the site and such control strategies, whether applied at a farm or an abattoir level could significantly benefit human health (Nayler et al., 2005).

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