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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 diarrhoea 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_2 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_2 gene detection by PCR technique. *E. coli* O157:H7 was found in 6(3.92%) of 153 sheep. The bacteria were isolated from 5(3.34%) 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-Aljaro *et al*., 2004). The Stx family contains two major, immunologically non-cross-reactive groups called Stx_1 and Stx_2 (Nataro and Kaper, 1998). Epidemiological data suggest that Stx_2 is more important than Stx_1 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 McConky (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

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Yahya Tahamtan and Shahram Shekarforoush have done equal amount of work to produce this paper

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 (Stx_2).

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 Stx_2 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 swap were rubbed to an area of 25 cm^2 in three different high risk fecal contamination areas (neck, shoulder and abdomen) (Fig. 1) and aseptically placed into 15 mL modified trypticase-soy broth (Merck) supplemented with ceffexime (0.15 mg L^{-1}) and potassium tellurate (5 mg L^{-1}) (Sigma) (mTSB). 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 mTSB were processed in two steps:

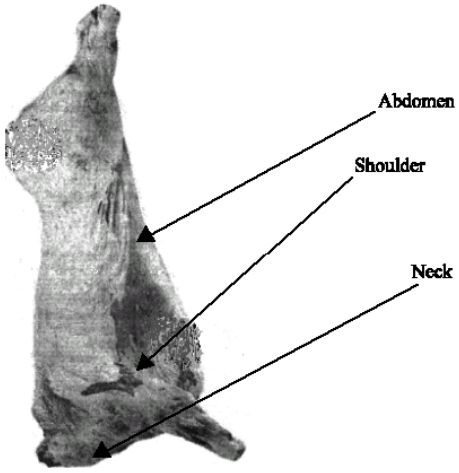

Fig. 1: The sampling areas of cattle and sheep carcasses (abdomen, shoulder and neck) (Bolton *et al*., 2001)

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 (Rogerie *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 (Rogerie *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 over night mTSB 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 (pH 9), 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 Stx_2 genes: To evaluate the presence of Stx_2 gene a pairs of primer for Stx_2 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 Stx_2 sequences were II-F_1 and II-R_2 that resulting in a PCR product of 779 bp, while for the nested system II-F_3 and II-R_4 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 Stx_2, 0.2 μM of each deoxynucleotide triphosphates, 1X PCR buffer (10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.001% gelatin), 1.5 mM $MgCl_2$, 2.5 U of thermostable DNA polymerase (Taq polymerase Roche Molecular system) and 2 μL DNA template for first PCR and 1 mM $MgCl_2$ and 0.3 μM of each Stx_2 primers for the second, Nested PCR with the primer pairs II-F_3/II-R_4. The reactions were carried out with a Techne Thermocycler (England) and cycling conditions with some modification were as follows: 94°C for 2 min, 30 cycle of 94°C for 30 sec, 57.5°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 analysed 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; Picozzi *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 10^8 to 10^1 colony forming unit (cfu) mL^{-1} were amplified without prior enrichment and also with overnight enrichment. The PCR procedure was down 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

Primers	Oligonucleotide sequence(5'3')	Product size (bp)
STII-F1	CCA TGA CAA CGG ACA GCA GTT	779
STII-R1	CCT GTC AAC TGA GCA CTT TG	
STII-F2	GTT CTG CGT TTT GTC ACT GT	372
STII-R2	AGC TGT ATT ACT TTC CCA TAA	

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).

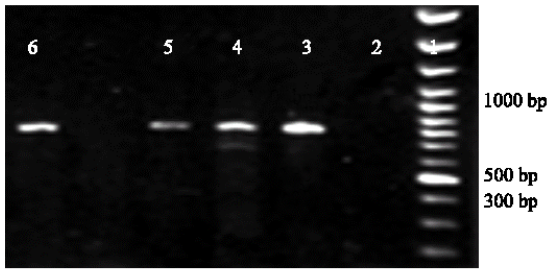

Fig. 2: Stx2 of *E. coli* O157. Lane 1: 100 bp ladder marker, Lane 2: Negative control, Lane 3-6: 779 bp positive samples

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

Abattoir No.	SN	MI	RP	UP	IP
1	5	4	2	4	3
2	3	3	1	2	2
3	3	2	2	1	3

SN: Sorbitol Negative, MI: Motile Isolate, RP: Rhamnose Positive, UP: Urease Positive, IP: Indol Positive

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

No. of animal source of isolate	Direct PCR(%)+	CT-SMAC (%) +	O157 antiserum positive+ (%)	PCR + (%)
153	6(3.92)[a]	11(7.18)[b]	7(5.57)[c]	4(2.62)[d]

Different superscripts letter(s) in each columns denote significant differences (p>0.026)

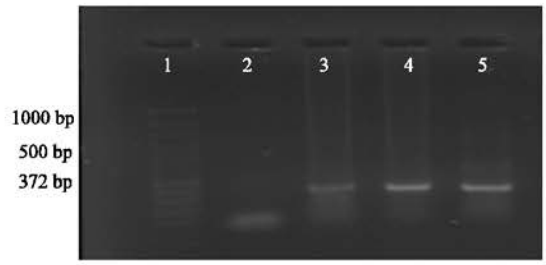

Fig. 3: Stx2 of *E. coli* O157. Products of Nested PCR on some *E. coli* O157:H7 isolates from sheep, Lane 1: 1000 bp ladder marker, Lane 2: Negative control, Lane 3-5: 372 bp positive samples

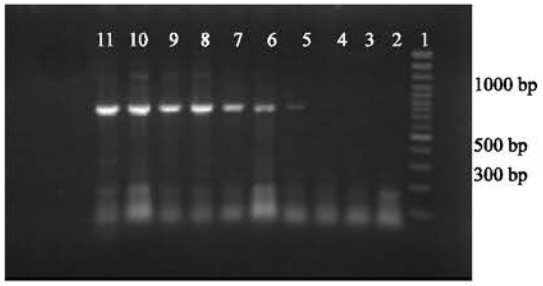

Fig. 4: Stx2 of *E. coli* O157. PCR products of a 10-fold serial dilution of *E. coli* O157:H7 (Without enrichment). Lane 1: 1000 bp ladder marker, Lane 2: Negative control, Lane 3-11: Bacterial suspensions containing 10^1 to 10^9 cfu mL^{-1}, respectively

Table 4: Isolation rate of *E. coli* O157:H7 from sheep carcasses at Shiraz slaughterhouse according to different ages (2005-2006)

Age	No. of animal source of isolate	No. (%) of positive cases
<=2	115	5 (3.34)[a]
>2	38	1 (2.26)[a]

Same superscripts letter(s) in each row denote no significant differences ($p>0.5$)

Table 5: Sensitivity of PCR systems alone and in combination with enrichment in mTSB of *E. coil* O157:H7 (without and with over night cultivation in mTSB)

Detection procedure	Detection limit
PCR alone*	10^3
Enrichment+PCR**	10^2

*PCR alone was done with suspension of mTSB, **PCR was done with suspension of mTSB after over night enrichment

Sensitivity and specificity of PCR and Nested PCR System: Sensitivity was same for both the systems, enabling the detection of DNA corresponding to about 10^3 and 10^2 cfu mL^{-1} (Table 5). As outlined in Fig. 4, detection was possible up to a dilution of 1×10^3 cfu mL^{-1} without prior enrichment and the use of an overnight enrichment step enhanced the detection limit by a factor of 100, enabling the detection of 10^2 cfu mL^{-1} (Fig. 5). The data indicate that the PCR systems are specific, with the length of the products being as expected, 779 bp for the

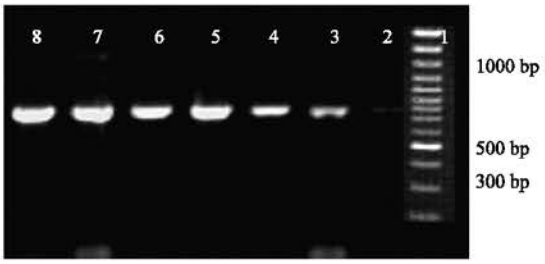

Fig. 5: Stx2 of *E. coli* O157. PCR products of a 10-fold serial dilution of *E. coli* O157:H7 (After overnight enrichment). Lane 1: 100 bp ladder marker, Lane 2: Negative control, Lane 3-8: Bacterial suspensions containing 10^2 to 10^8 cfu mL^{-1}, respectively.

Stx_2 PCR and 372 bp for the Stx_2 Nested PCR systems. No amplicons were obtained from strains that did not contain the Stx_2 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 human (CDC, 1995). It is important to know the contamination during slaughter since the majority of food-borne *E. coli* O157:H7 infections in human 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 Stx_2 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 4.0% 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_2 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 (Naylor *et al*., 2005).

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