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Original Article

Detection of Shiga toxin-producing *Escherichia coli* (STEC) in faeces of healthy calves in Mashhad, Iran

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ABSTRACT

The aim of this study was to identify virulent Shiga toxin-producing *Escherichia coli* (STEC) strains isolated from faecal samples of 100 clinically healthy calves. In the present study, a total of 100 *Escherichia coli* (*E. coli*) isolates from clinically healthy calves belonging to 6 different farms located in Khorasan Razavi province, Iran, were examined for presence of virulence genes characteristic for STEC strains. Duplex PCR assay was used in order to determine the presence of the *stx1*, and *stx2* genes. The *stx1* or *stx2* positive isolates was later analyzed with primers specific for *eaeA*, *hlyA* genes. PCR assay was also conducted on these isolates for detection of O157: H7 serotype. Our results showed that 15 isolates (15%) were positive for stx1 *gene*, 19 isolates (19%) were positive for stx2 gene and 8 isolates (8%) were positive for both *stx1*, and *stx2*. Totally 26 isolates were positive for at least one of *stx1*or *stx2* genes. Among 26 isolates, which were tested for the presence of *eaeA* and *hlyA* genes, 5 and 22 of them were positive for these genes, respectively. Four isolates were positive for both *eaeA* and *hlyA* genes. Among 26 isolates which were analyzed for genes coding O157 and H7 antigens, one and four isolates were positive for O157 and H7 antigen gene, respectively. None of the isolates were positive for both antigen genes. Faecal contamination due to poor hygiene is a risk factor in contamination of meat and milk products.

Keywords: Shiga toxin, Escherichia coli O157:H7, stx1, stx2, eae, hly

INTRODUCTION

Shiga-toxin-producing Escherichia coli (STEC) are a subgroup of *Escherichia coli* that cause very important deadly diseases in humans (Griffin 1995). First identification of *E. coli* O157 as a food-borne pathogen was in 1982 (Riley & Remis 1983). *Escherichia coli* O157: H^- and O157:H7 are members of STEC and has emerged as an important enteric pathogen posing a remarkable challenge to public health (Cagney *et al* 2004). Illnesses caused by STEC strains can range from mild, watery non-bloody diarrhea to life-threatening conditions, such as haemolytic uremic syndrome (HUS), haemorrhagic colitis (HC) and thrombotic

thrombocytopenic purpura (TTP) (Coia, 1998). Infectious dose of these pathogens may be as low as 10 organisms (Cagney *et al* 2004). The pathogenicity of STEC strains including O157: H^- and O157:H7 serotypes, is associated with several virulence factors. The STEC typically contains genes encoding these virulence factors mainly shigatoxins (*stx1* and/ or *stx2*), intimin proteins (*eaeA*), and enterohaemolysin (*hlyA*) (Tesh & O'Brien 1991). HC and HUS diseases are caused by Shiga-toxins. These toxins inactivate ribosomal RNA, inhibit protein synthesis and cause the death of host cells (O'Brien & Holmes 1987). They have cytopathic effects on the vascular endothelial cells of the intestines, kidneys, the central nervous system, and other organs. Intimin is a protein encoded by the *eaeA* gene, expressed by STEC strains and is responsible for the intimate attachment of the STEC to the intestinal epithelial cells. This further causes attaching and effacing lesions in the intestinal mucosa (Garrido *et al* 2006). Another virulence factor of STEC strains is haemolysin which is encoded by the *hlyA* gene. This haemolysin seems to have some important role in complex diseases (HC and HUS) in humans, as reported in a study, 90% of diseases associated with STEC strains, carried a haemolysin encoding gene (Beutin *et al* 1994). STEC strains may be transmitted to human through numerous ways, especially through the consumption of faecally contaminated food or water, person to-person or animal-to-person contact (Ackers et al., 1998; Heuvelink et al., 1999). Domestic animals like cattle or sheep have been shown not only to sporadically excrete STEC, but also to maintain their colonization with STEC for very long periods (Liebana *et al* 2005, Sánchez *et al* 2009, Shere *et al* 1998). One of the main natural reservoirs of *E. coli* O157:H7 are Cattles, but generally domestic animals have been demonstrated that excrete *E. coli* O157: H^- and *E. coli* O157:H7 (Heuvelink *et al* 1998b, Hancock *et al* 1998). The pathogen has been shown to occur in the faeces, rumen, hides, and derived carcasses (Cagney *et al* 2004). Regarding human food chain, numerous studies have reported the presence of *E. coli* O157:H7, usually at low prevalence on retail meats (Cagney *et al* 2004, Carney *et al* 2006, Çadırcı *et al* 2010, Ojo *et al* 2010). The aim of present study was to identify STEC isolated from faecal samples of 100 clinically healthy calves and investigation of enterohaemolysin (*hlyA*) and intimin (*eaeA*) virulence genes in these bacteria. Also, the presence of *E. coli* O157:H^- and *E. coli* O157:H7 serotypes were investigated among isolated STEC.

MATERIALS AND METHODS

Bacterial Isolates and DNA Extraction. A number of 100 Escherichia coli isolates were obtained from department of microbiology, School of Veterinary Medicine, Ferdowsi University of Mashhad, Iran. They were isolated from faecal samples of 100 clinically healthy calves using conventional culture methods. The age of calves was under one year old. Genomic DNA of bacterial cells was extracted by the use of boiling method. Isolated bacteria were subcultured on tryptone soy agar. Typical colonies were harvested and then suspended in 250 µl of deionized and sterilized distilled water. Microtubes containing bacterial suspension were incubated at 100 °C for 10 min, and then centrifuged at 15000 rpm for 15 minutes. The supernatants were transferred to sterile nuclease free microtubes and freezed at -18 °C until use.

Detection of virulence genes and O157 and H7 genes by PCR. In the present study, 100 *E. coli* isolates from clinically healthy calves were examined for determination of STEC. Duplex PCR was used for detection of the *stx1*, *stx2* genes (AssayI), *eaeA*, *hlyA* genes (Assay II) and *rfbEO157*, *fliCh7*genes (Assay III). The sequences of the primers which were used in this study and predicted sizes of the amplified products are shown in Table 1. The *stx1* and *stx2* positive isolates were later analyzed with primers specific for *eaeA*, *hlyA* and *rfbEO157*, *fliCh7*genes. Amplification of bacterial DNA was performed using premix PCR Kit (20 µl volumes) (Bioneer, Southern Korea). Every reaction contained 10 picomol of each oligonucleotide primers, 2µl of the DNA template, and 14 µl deionized distilled water. PCR reaction was performed in a

thermal cycler (model Techne, Germany). The PCR condition was carried out as follows: 94 °C for 5 min for initial denaturation, followed by 35 cycles of 94 °C for 1 min, 55 °C for 30 seconds, and 72 °C for 1 min and a final extension of 72 °C for 10 min. *E.coli* O157:H7 (ATCC 43895) was used as a positive control and deionized distilled water for negative control. Four microlitres of PCR products were prestained by etidium bromide and run on 1% agarose gel and were visualized under UV illumination.

RESULTS

In this study, 100 *E. coli* isolates from faeces of healthy calve were examined for STEC virulence genes by PCR. 15 isolates (15%) were positive for stx1 *gene*, 19 isolates (19%) were positive for stx2 gene and 8 isolates (8%) were positive for both of them (Figure 1).

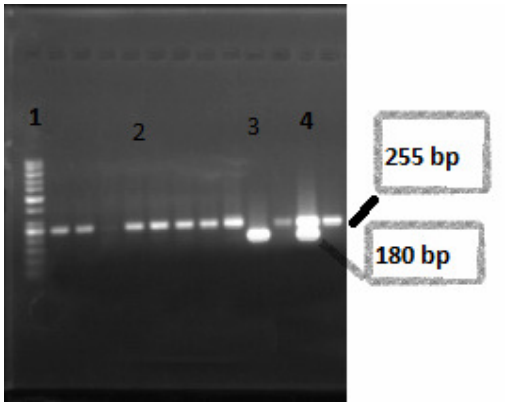

Figure 1. Agarose gel (1%) of Stx1 and *stx2* gene fragments that were amplified from DNA extracted from the STEC strains and E. coli control strain. Lane 1 DNA marker (50 base pairs); Lane 2 stx2 positive sample; Lane 3 stx1 positive sample and Lane 4 positive control.

Totally 26 isolates were positive for at least one of the stx1 or stx2 genes (STEC strains). These 26 isolates were tested for the presence of *eaeA* and *hlyA* genes. According to our results 5 and 22 isolates were positive for *eaeA* and *hlyA* genes, respectively and 4 out of 26 isolates were positive for both of these genes (Figure 2). The third duplex PCR assay was also conducted on these isolates for detection of O157: H7 serotype. Among 26 isolates that were analyzed for the presence

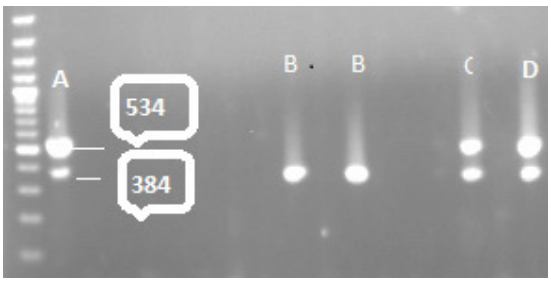

Figure 2. Agarose gel (1%) of *eaeA* and *hlyA* gene fragments that were amplified from DNA extracted from the STEC strains and E. coli control strain with DNA marker (100 base pairs); Lane A positive control; Lane B *eaeA* positive samples; Lane C & D *hlyA* positive samples.

of O157 and H7 antigen genes, one isolate was positive for O157 antigen gene and four isolates were positive for H7 antigen's gene, whereas none of the isolates were positive for both of them. In our study one O157 positive isolate and four H7 positive isolates were detected (Figure 3).

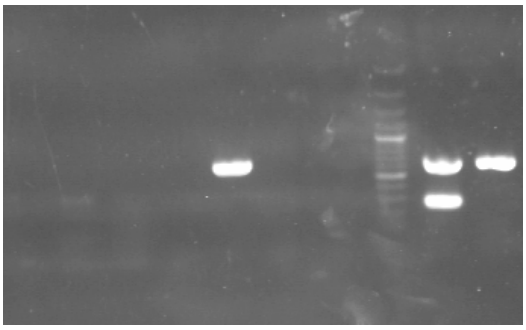

Figure 3. Agarose gel (1%) of O157 and *flic*H7 gene fragments that were amplified from DNA extracted from the STEC strains and E. coli control strain. Lane 1 Negative control; Lanes 2, O157 positive samples; Lanes 5 & 10 H7 positive sampls; Lanes 3,4, 6, and 7 negative samples; Lane 8 DNA marker (100 base pairs); Lanes 9 positive control E. coli O157:H7 (ATCC 43895).

DISCUSSION

Domestic animals are the main reservoirs of O157:H7 serotypes, but their occurrence is likely to be underestimated, because isolation of nonO157:H7 STEC still remains a challenge. The majority of non-O157 strains ferment sorbitol and therefore cannot be identified by using media such as sorbitol MacConkey agar. Many studies have been conducted at the abattoir

Table 1. Sequence of primers and the size of amplified products

	Primer	Sequence (5´-3´)	Reference	Amplification product size (bp)
Assay I	*stx1*F	ATAAATCGCCATTCGTTGACTAC	Paton and Paton (1998) modified by Fitzmaurice et al. (2004)	180
	*stx1*R	AGAACGCCCACTGAGATCATC		
	*stx2*F	GGCACTGTCTGAAACTGCTCC	Paton and Paton (1998) modified by Fitzmaurice et al. (2004)	255
	*stx2*R	TCGCCAGTTATCTGACATTCTG		
Assay II	*eaeA*F	GACCCGGCACAAGCATAAGC	Paton and Paton (1998)	384
	*eaeA*R	CCACCTGCAGCAACAAGAGG		
	*hlyA*F	GCATCATCAAGCGTACGTTCC	Paton and Paton (1998)	534
	*hlyA*R	AATGAGCCAAGCTGGTTAAGCT		
Assay III	*rfbeO157*F	CGGACATCCATGTGATATGG	Paton and Paton (1998)	259
	*rfbeO157*R	TTGCCTATGTACAGCTAATCC		
	fliCh7-F	GCGCTGTCGAGTTCTATCGAGC	Fratamico et al. (2000)	625
	fliCh7-R	CAACGGTGACTTTATCGCCATTCC		

level on cattle carcasses and their feces for determination of the prevalence of *E. coli* O157:H$^-$ or *E. coli* O157:H7 and their virulence genes (Carney *et al* 2006, McEvoy *et al* 2003, Yilmaz *et al* 2006). Carney et al., (2006) was recovered E. coli O157 from 2.4%, of beef trimmings samples. 96.87% contained the eaeA and hylA genes while 93.75% contained the fliCh7 gene and 96.87% contained vt1 or vt2, or both vt genes (Carney *et al* 2006). E. coli O157 was recovered from 3.0% of both carcass samples and head meat which all of them contained the eaeA, hylA and fliCh7 genes (Carney *et al* 2006). While all of the head meat samples contained vt2 genes, but no one contained the vt1 gene (Carney *et al* 2006). McEvoy et al. (2003) were examined the prevalence of E. coli O157:H7 and their genes encoding verocytotoxin, enterohaemolysin and intimin production on carcasses and bovine feces at the abattoir. E. coli O157:H7 was isolated from 14.11% and 2.4% of carcasses and bovine feces, respectively. Yilmaz, et al. (2006) showed that eaeA gene was detected in all O157 and O157:H7 strains tested. Both VT2 and eaeA genes were detected in 80% of strains of E. coli O157 (Yilmaz *et al* 2006). Our results showed that 15 (15%) and 19 (19%) isolates were positive for *stx1* and *stx2* gene, respectively. A number of 8 isolates (8%) were positive for both of *stx1* and *stx2* genes. Blanco et al. (2003) reported that 42% of their isolates were positive for both of these genes. Different rates of presence of these toxins have been reported in healthy and diarrheic calves (Blanco *et al* 2001). 32.7% of STEC strains from diarrheic calves in Vietnam carried genes for *stx1* and *stx2*. 25.9% and 41.24% of these strains were positive for *stx1* and *stx2* genes, respectively (Nguyen *et al* 2011). However, in other study, none of the *E. coli* isolates from healthy calves were positive for the genes associated with these toxins (Ok *et al* 2009). Blanco et al. (2001) emphasized that while the prevalence of *stx1* was higher in diarrheic cattle, *stx2* or both *stx1* and *stx2* were predominant among STEC strains from healthy cattle. Similar results were reported by Wieler et al. (1992) in Germany, after characterizing STEC strains isolated from calves with diarrhoea and without diarrhoea. They reported that a significantly higher percentage of stx1-producing *E. coli* was found in diarrheic calves. Also, in the present study, presence of stx2 genes was higher than stx1 alone or *stx1* and *stx2* positive isolates. In a study by Aslantas et al. (2006) in Turkey, among the 77 STEC strains isolated from Turkish cattle, 62 and 3 of them were found to be positive for *stx2*, and both *stx1*/*stx2*

genes, respectively. A strong association was reported between pathogenesis of STEC strains in human disease and the presence of *eaeA* gene (Beutin *et al* 2004). According to different studies, detection of *eaeA* gene is differed among STEC from healthy or diarrheic cases (Sandhu *et al* 1996, Aidar-Ugrinovich *et al* 2007). The low prevalence of *eaeA* gene is a characteristic of STEC isolates from healthy cattle (Wieler *et al* 1996). The results of the present study are according to this due to only 5 out of 26 STEC isolates were positive for *eaeA* gene. Moreover, all of *eaeA* positive isolates were also producer of *stx1*. This finding is in accordance with a study by Sandhu et al. (1996). Enterohaemolysin is a protein encoded by *hlyA* gene and has relatively important role in the pathogenesis of STEC. It may be lead to enterocyte and leukocyte lyses in cattle (Bauer & Welch 1996). In this study, *hlyA* was the most common virulence gene. Aidar-Ugrinovich et al. (2007) observed that 25% and 58% of STEC strains from healthy calves were positive for *eaeA* and *hlyA* genes, respectively. Among 26 STEC isolates which were analyzed for genes coding O157 and H7 antigens, 1 and 4 isolates were positive for O157 and H7, respectively. None of the isolates were positive for both of them. Ateba et al. (2011) observed that among 5600 presumptive *E. coli* O157:H7, 130 isolates were positive for O157 and H7 encoding genes. Also, the presence of *hlyA* gene (56.2%) was higher than *eaeA* gene (36.9%) that is consistent with our results. However, in our study *E. coli* O157:H$^-$ isolate harbored only stx2 and *hlyA* genes, that is in agreement with finding of Ateba et al. (2011). Most of the *E. coli* O157 isolates recovered from farm animals regularly harbors *eaeA* and *stx2* genes, but not the *stx1* gene (McEvoy *et al* 2003). There are variable results regarding the presence of virulence genes (*hlyA, katP, espP*) in *E. coli* O157:H7 and STEC strains isolated from cattle. However, strains lacking one or more of these genes have been involved in HUS (Brunder *et al* 1999). Rogerie et al. (2001) observed that five of eight *E. coli* O157 strains had no virulence gene at all, and two strains were positive for *eae*A and *hly*A genes, while the other one had only *eae*A gene. Aslantas et al (2006) reported 12 *E. coli* O157:H7 strains which were *stx* negative. Although *stx* genes are generally considered to be essential for the pathogenesis of *E. coli* O157:H7, Schmidt et al. (1999) reported that no Shiga toxin-producing *E. coli* O157:H7 strains can cause HUS. STEC strains in calves may be important due to ability of some strains to produce diarrhea and lead to economical loss. But, in human, they are very important because of some deadly disease which were produced. These animals could be carriers for human. Cattle are considered to be the major reservoir of STEC worldwide (Aidar-Ugrinovich *et al* 2007).

Regarding the results of the present study, STEC other than O157:H7 present in healthy calves so healthy calves can act as important reservoirs of STEC for human. Development of strict sanitary condition for food animal husbandry and derived products are one of the main approaches to decrease the dissemination of these pathogens.

Ethics

I hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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