Prevalence of *Escherichia coli* O157:H7 on dairy farms in Shiraz, Iran by immunomagnetic separation and multiplex PCR

Sami, M.[1*]; Firouzi, R.[2] and Shekarforoush, S. S.[3]

[1]Department of Food Hygiene and Public Health, School of Veterinary Medicine, Shahid Bahonar University of Kerman, Kerman, Iran; [2]Department of Pathobiology, School of Veterinary Medicine, University of Shiraz, Shiraz, Iran; [3]Department of Food Hygiene and Public Health, School of Veterinary Medicine, University of Shiraz, Shiraz, Iran

[*]**Correspondence:** M. Sami, Department of Food Hygiene and Public Health, School of Veterinary Medicine, Shahid Bahonar University of Kerman, Kerman, Iran. E-mail: sami@mail.uk.ac.ir

(Received 25 Jun 2006; revised version 17 Sept 2006; accepted 30 Oct 2006)

Summary

To identify the reservoirs of shiga toxin-producing *Escherichia coli* O157, sensitive detection and isolation methods are necessary. The sensitivity of traditional culture methods can be improved significantly by the inclusion of an immunoconcentration step, resulting in less false-negative results. In this study, enrichment procedure and immunomagnetic separation (IMS) were compared for use in conjunction with a multiplex polymerase chain reaction (M-PCR) method for detection of genes *stx*$_1$, *stx*$_2$, *eae*A and *hly*A. A total number of 975 faecal samples were collected from 26 dairy farms in Shiraz area, Shiraz, southern Iran. The samples were cultured at 37°C for 18–24 hrs in modified tryptic soy broth (m-TSB). Each of five enriched samples were pooled and examined in two ways—direct PCR and IMS. The detection limit of the M-PCR protocol for seeded *E. coli* O157:H7:ATCC:43895 in m-TSB without stool was 1.23×10^2 CFU/ml, whereas it was 1.23×10^6 CFU/ml with enriched faecal sample. In direct PCR of enriched samples, no positive sample was detected. However, in IMS of enriched samples one specimen was positive. The prevalence of *E. coli* O157:H7 in faeces of cows in examined farms was 0.51% and the herd prevalence was 3.86%. Isolation of this serotype from faecal samples indicates that cattle are reservoirs of this pathogen and potentially a source of human infection. This finding is of considerable public health importance.

Key words: *Escherichia coli* O157:H7, Cattle, M-PCR, IMS, Shiraz

Introduction

Enterohemorrhagic *E. coli* (EHEC) is an important group of food-born pathogens. EHEC, including *E. coli* O157:H7 comprise a subset of shiga toxin-producing *E. coli* (STEC). *E. coli* O157:H7 has been known to be a human pathogen for nearly 24 years (Riley *et al*., 1983). Cattle are considered to be the major reservoir of *E. coli* O157:H7 infection for man, although the organism has also been isolated from sheep, goat, deer, horses, dogs, birds and flies (Whipp *et al*., 1994; Hancock *et al*., 1998). The pathogenicity of *E. coli* O157:H7 is associated with several virulence factors including shiga toxins or verotoxins 1 and 2 (encoded by genes *stx*$_1$ and *stx*$_2$), which cause haemorrhagic colitis (HC) and haemolytic-uremic syndrome (HUS). The ability to adhere to the intestinal mucosa by intimin (encoded by the *eae*A gene) and the production of haemolysin (encoded by the *hly*A gene) which lyses erythrocytes, would release heme and haemoglobin that enhance the growth of *E. coli* O157:H7 and that could serve as a source of iron (Nataro and Kaper, 1998). Most strains of *E. coli* O157:H7 are unable to ferment D-sorbitol, and thus distinguishing them from many other faecal *E. coli* is not difficult. Specimens are therefore, plated on MacConkey agar containing 1% D-sorbitol instead of lactose (SMAC). Addition of both cefixime and potassium tellurite improves selection of *E. coli* O157:H7 over other *E. coli* and non-

sorbitol fermenters such as *Aeromonas*, *Plesiomonas*, *Morganella* and *Providencia* (Chapman *et al*., 1991; Heuvelink *et al*., 1997). Rapid and reliable methods to detect *E. coli* O157:H7 are important to identify the source of outbreaks and to assure public safety. Because of the small numbers of *E. coli* O157 present in many stool samples and food stuff, immunomagnetic separation (IMS) with commercially available magnetic beads coated with antibody against O157 antigen has been used (Chapman *et al*., 1994).

The objective of this study was to identify and to estimate the prevalence of *E. coli* O157:H7 in dairy cattle faecal samples using a rapid and specific method. Isolation of *E. coli* O157:H7 was achieved by enriching the organism in broth culture, isolating *E. coli* O157:H7 from other organism by immunomagnetic cell separation and then culturing captured cells on selective media and detecting them using multiplex polymerase chain reaction (M-PCR).

Materials and Methods

Sample collection

A total number of 975 faecal samples were collected from milking cows of 26 dairy farms in Shiraz area, southern Iran. The samples were collected from rectum or as fresh faecal samples from the bedding. The farms divided into two groups—small farms with ≤50 and large farms with >50 lactating cows. According to herd size, 30–50 samples were collected from each farm. Samples were immediately transported to the laboratory.

Sample enrichment

The samples were cultured on modified tryptic soy broth (m-TSB) containing cefixime 0.05 mg/l and incubated at 37°C for 18–24 hrs (Oksuz *et al*., 2004). Each of five enriched samples were pooled and examined as follows:

DNA extraction

One ml of the enriched mixture was transferred to a clean microtube and centrifuged at 14,000 × g for one min. The pellet was resuspended in 500 µl of 0.85% NaCl, and centrifuged for one min. Then, the pellet was resuspended in 250 µl of distilled water. After boiling for 10 min, the samples were chilled immediately. An aliquot of 10 µl of the supernatant was collected as a PCR template without centrifugation (Holland *et al*., 2000).

IMS

IMS was carried out on one-ml aliquots of the pooled enriched sample. Twenty µl of immunomagnetic beads (Dynal, Norway) was added to microcentrifuge tubes. Sample and beads were placed in a rotating mixer for 30 min at room temperature. The tubes were then placed in a magnetic rack and the beads were drawn to the side of the tube. After discarding the supernatant, tubes were removed from the magnetic rack and the beads were washed with one ml of sterile tween-PBS mixture. The washing step was repeated three times. Finally, the beads resuspended in 100 µl of sterile tween-PBS. An aliquot of 25 µl of the solution was then plated on SMAC agar containing 0.05 mg/l cefixime and 2.5 mg/l potassium tellurite and incubated at 37°C for 18–24 hrs (Chinen *et al*., 2003). All suspected colonies (non-sorbitol fermenter) were collected for M-PCR evaluation.

M-PCR assay

For determination of *stx*$_1$, *stx*$_2$, *eae* and *hly* genes, the designed primers (Table 1) and PCR conditions were optimized for M-PCR using recommendations reported previously with some modifications (Paton and Paton, 1998). M-PCRs were performed with 25 µl reaction mixtures containing 0.2 µl of Taq DNA polymerase (Fermentase, 5U/µl), 2.5 µl of 10X PCR buffer, 3 µl $MgCl_2$ (CinnaGen Inc. 50 mM), 0.5 µl of dNTPs (CinnaGen Inc. 10 mM), 0.5 µl of each of eight primers (TIB Molbiol, 20 pmol/µl), 10 µl DNA template and 4.8 µl sterile deionized water to bring the total volume of the PCR reaction mixtures to 25 µl. The positive control contained 10 µl of *E. coli* O157:H7:ATCC:43895 DNA and the negative control contained water instead of DNA in the PCR mixture. Reaction mixtures were held in a thermocycler (GENIUS-

Table 1: Primers used for M-PCR (Paton and Paton, 1998)

Primer	Target	Sequence (5′-3′)	Specificity	Amplicon size
stx$_1$F	*stx$_1$*	ATAAATCGCCATTCGTTGACTAC	nt 454-633	180 bp
stx$_1$R	*stx$_1$*	AGAACGCCCACTGAGATCATC	nt 633-454	
stx$_2$F	*stx$_2$*	GGCACTGTCTGAAACTGCTCC	nt 603-857	255 bp
stx$_2$R	*stx$_2$*	TCGCCAGTTATCTGACATTCTG	nt 857-603	
eaeAF	*eae*A	GACCCGGCACAAGCATAAGC	nt 27-410	384 bp
eaeAR	*eae*A	CCACCTGCAGCAACAAGAGG	nt 410-27	
hlyAF	*hly*A	GCATCATCAAGCGTACGTTCC	nt 70-603	534 bp
hlyAR	*hlyA*	AATGAGCCAAGCTGGTTAAGC	nt 603-70	

nt: nucleotide

TECHNE-UK). The M-PCR cycling condition included an initial denaturation at 95°C for two min followed by 35 cycles, with one cycle consisting of one min at 95°C, two min at 62°C and 1.5 min at 72°C with a final chain elongation at 72°C for seven min. Five µl of each PCR product were analysed on 2% agarose gel containing 0.1 µl/ml ethidium bromide (CinnaGen Inc. 10 mg/ml) with the 50 bp DNA ladder plus, as a size marker and visualized under UV illumination.

Sensitivity of M-PCR

To assay the sensitivity of M-PCR, one of the VTEC negative faecal cultures was spiked with 10-fold serial dilutions of *E. coli* O157:H7:ATCC:43895.

In a parallel exam, serial 10-fold dilution in m-TSB without stool was prepared from culture of *E. coli* O157:H7. It was estimated from direct plate count that the initial broth culture of strain O157:H7 contained 1.23×10^7 CFU/ml. Then one ml of this culture was added to nine ml of the first tube of seven replicate dilution series. Each of these dilutions was used for M-PCR detection.

Specificity of M-PCR

To determine the specificity of the M-PCR protocol, six different bacterial strains included *Citrobacter* spp., *Pseudomonas* spp., *E. coli* serotype K12, *Staphylococcus aureus*, *Lactobacillus* spp. and *Enterococcus faecalis* were tested by M-PCR with the same conditions described above.

Results

A total number of 195 pooled faecal samples were analysed by *stx*$_1$, *stx*$_2$, *eae* and *hly* specific gene primers. In direct PCR of enriched samples, no positive specimen was obtained. In IMS method, 44 suspected colonies were isolated in which only one specimen, from a dairy farm with >50 lactating cows, was positive for *E. coli* O157:H7 (Fig. 1). The amplicons were sequenced, and results confirmed with Gene bank O157 sequences (data not shown). The prevalence of *E. coli* O157:H7 in faeces of cows in examined farms was 0.51% and the herd prevalence in Shiraz, Iran was 3.86%.

In assessment of the sensitivity of M-PCR, the detection limit of PCR procedure for seeded *E. coli* O157:H7 strain was 1.23×10^6 CFU/ml of faecal culture. However, the limit value for seeded *E. coli* O157:H7 strain in m-TSB without stool was 1.23×10^2 CFU/ml (Fig. 2).

Using primers set for amplification of the 180, 255, 384 and 534 bp products, amplicons were observed just for standard strain of *E. coli* O157:H7 but not for other bacterial strain (Fig. 3).

Discussion

E. coli O157:H7 increasingly becomes as a cause of serious disease in human with asymptomatic animal carriers as a reservoir of infection. Carrier animal excretes the organism intermittently and in low numbers in faeces.

Cattle and their faeces have been considered as the primary source of VTEC, and the reported incidence of *E. coli* O157 in cattle faeces varies from 0.1% (Johnsen *et al*., 2001) to 62% (Jackson *et al*., 1998). PCR is generally accepted as the most sensitive means of determining whether a faecal or a food sample contains VTEC. Although direct extracts of faeces or foods can be used as templates for PCR, the best

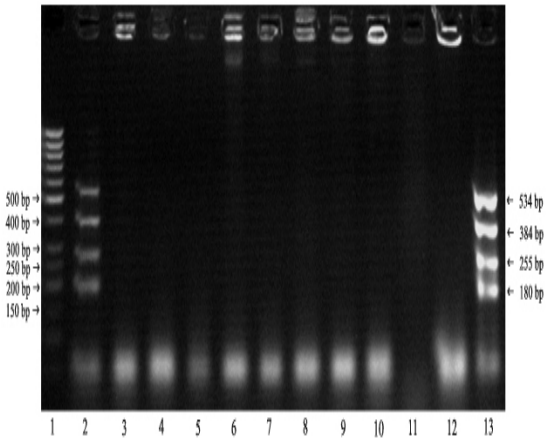

Fig. 1: PCR products of some faecal samples. Lane 1: 50 bp DNA ladder; Lane 2: positive control; Lane 3: negative control; Lane 4–12: negative samples and Lane 13: positive sample

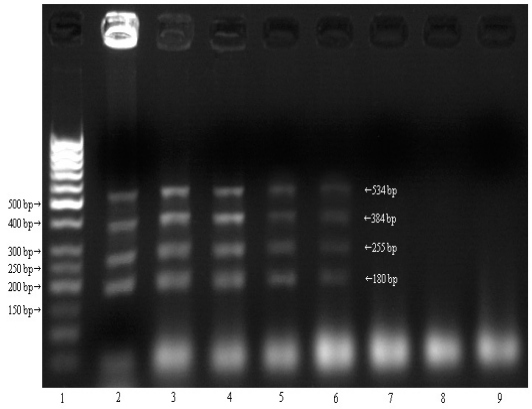

Fig. 2: PCR products of a 10-fold serial dilution of *E. coli* O157:H7 culture. Lane 1: 50 bp DNA ladder; Lane 2–8: bacterial suspensions containing 1.23 × 10^6 to 1.23 CFU/ml, respectively and Lane 9: negative control

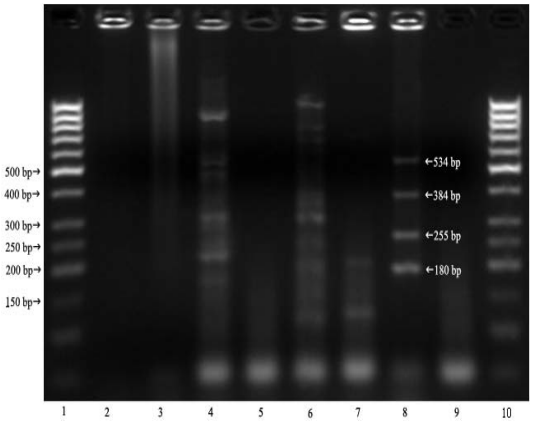

Fig. 3: PCR products of 7 bacterial strains. Lane 1 and 10: 50 bp DNA ladder; Lane 2: *Citrobacter* spp.; Lane 3: *Pseudomonas* spp.; Lane 4: *E. coli* serotype K12; Lane 5: *S. Aureus;* Lane 6: *Lactobacillus* spp.; Lane 7: *Enterococcus faecalis*; Lane 8: *E. coli* O157:H7 and Lane 9: negative control

result is usually obtained by testing extracts of primary broth culture (Paton *et al*., 1993). False-negative PCR results may be due to the presence of a small number of target organisms in the volume of stool sample and the decreased stability of cell with storage (Da Silva *et al*., 1999). In this study no *E. coli* O157:H7 strain was detected in enriched faecal samples with direct PCR. Different types of faecal inhibitors such as haemoglobin degradation products, bilirubin, bile acid and etc. contract with direct PCR results (Nataro and Kaper, 1998). However in IMS, in addition to specific cultural method, one specimen identified as *E. coli* O157:H7. IMS improves the isolation of *E. coli* O157:H7 from cultures containing low numbers of organism. IMS has been shown to be efficient in detecting 10^2 CFU of *E. coli* O157 per each gram of faeces compared to the presence of 10^5 CFU/g faeces of organism required for detection by PCR (Chapman *et al*., 1994; Clifton-Hadley, 2000).

A one-year study at an abattoir in northern England revealed a prevalence of 15.7% *E. coli* O157 in cattle prior to slaughter with the highest isolation rate in spring and late summer (Chapman *et al*., 1997). In a similar study in Turkey, *E. coli* O157 was isolated from 13.6% of faecal samples with the highest occurrence in July and November and the lowest in February (Aslantas *et al*., 2006). In a nation-wide Dutch study, 10.6% of faecal sample from adult cattle collected from the major abattoirs were positive for *E. coli* O157 (Heuvelink *et al*., 1998). These investigators reported the occurrence of *E. coli* O157:H7 most frequently in 1- to 3-year-old cattle. The higher frequency in younger animals may be due to differences in the composition of the gastrointestinal flora resulting from differences in diet (Heuvelink *et al*., 1998).

Low isolation rate of *E. coli* O157:H7 in this study could be attributed to several factors such as geographic distribution, age, breed and housing condition.

Other studies have shown similar rates of isolation (Chapman *et al*., 1994; Lahti *et al*., 2001). Another reason for low prevalence rate observed in our study could be sampling during the cold months, which

is in agreement with the findings of other studies on seasonal variation of the infection with more cattle being found positive for VTEC O157 in late summer and early autumn (Chapman *et al*., 1997; Aslantas *et al*., 2006). Dontorou *et al*. (2004) reported a rare isolation of *E. coli* O157:H7 strain from goat faeces with no isolates from other farm animals including sheep and cattle in Greece. Nielsen *et al*. (2002) reported the effect of age (2–6 month) as a high risk factor with no influence of herd size, housing condition and sex. This phenomenon is likely to be related to the immature rumenal fermentation in the veal calves in combination with the highly acidic environment of the more developed abomasal flora (Nielsen *et al*., 2002). Furthermore, a new research was indicated that pooling faecal sample decreased the sensitivity of detection at low prevalence compared to individual samples, but the sensitivity was similar at high prevalence rates (Sanderson *et al*., 2005). In the present study, pooling was performed after enriching the faecal samples. Pooled faecal samples may provide the ability for scanning herds more rapidly and at lower costs (Sanderson *et al*., 2005). In addition, the *stx*, *eae* and *hly* genes could be easily detected in 10^2 CFU after enrichment of bacterial population followed by IMS, and M-PCR.

In conclusion, the isolation of one strain from faecal samples indicates that cattle are reservoirs of this pathogen and potentially a source of human infection. This finding is of considerable public health importance.

Acknowledgements

This work was financially supported by the office of Vice Chancellor for Research of Shiraz University. The authors would appreciate Dr. M. Ansari, Ms. M. Masoudian, Mr. A. Saeedzadeh and Mr. G. A. Niknia for their assistance.

References

Aslantas, O; Erdogan, S; Cantekin, Z; Gulact, I and Evrendilek, GA (2006). Isolation and characterization of verocytotoxin-producing *E. coli* O157 from Turkish cattle. Int. J. Food Microbiol., 106: 338-342.

Chapman, PA; Siddons, CA; Cerdan Malo, AT and Harkin, MA (1997). A 1-year study of *Escherichia coli* O157:H7 in cattle, sheep, pigs and poultry. Epidemiol. Infect., 119: 245-250.

Chapman, PA; Siddons, CA; Zadik, PM and Jewes, L (1991). An improved selective medium for the isolation of *Escherichia coli* O157. J. Med. Microbiol., 35: 107-110.

Chapman, PA; Wright, DJ and Siddons, CA (1994). A comparison of immunomagnetic separation and direct culture for the isolation of verotoxin-producing *Escherichia coli O157* from bovine faeces. J. Med. Microbiol., 40: 424-427.

Chinen, I; Otero, JL; Miliwebsky, ES; Roldan, ML; Baschkier, A; Chillemi, GM; Noboli, C; Frizzo, L and Rivas, M (2003). Isolation and characterisation of shiga toxin-producing *Escherichia coli* O157:H7 from calves in Argentina. Res. Vet. Sci., 74: 283-286.

Clifton-Hadley, FA (2000). Detection and diagnosis of *Escherichia coli* O157 and other verocytotoxigenic *E. coli* in animal faeces. Rev. Med. Microbiol., 11: 47-58.

Da Silva, AJ; Bornay-Liinares, FJ; Moura, IN; Slemenda, SB; Tuttle, JL and Pieniazek, NJ (1999). Fast and reliable extraction of protozoan parasite DNA from faecal specimens. Mol. Diagn., 4: 57-64.

Dontorou, A; Papadopoulou, C; Filioussis, G; Apostolou, I; Economou, V; Kansouzidou, A and Levidiotou, S (2004). Isolation of a rare *Escherichia coli* O157:H7 strain from farm animals in Greece. Comp. Immunol. Microbiol. Infect. Dis., 27: 201-207.

Hancock, DD; Besser, TE; Rice, DH; Ebel, ED; Herriott, DE and Carpenter, LV (1998). Multiple sources of *Escherichia coli* O157 in feedlots and dairy farms in the Northwestern USA. Prev. Vet. Med., 35: 11-19.

Heuvelink, AE; Van Den Biggellar, FLAM; De Boer, E; Herbes, RG; Melchers, WJG; Huis Intveld, JHJ and Monnens, LAH (1998). Isolation and characterization of verocytotoxin producing *Escherichia coli* O157 strain from Dutch cattle and sheep. J. Clin. Microbiol., 36: 878-882.

Heuvelink, AE; Zwartkruis-Nahuis, JTM and De Boer, E (1997). Evaluation of media and test kits for the detection and isolation of *Escherichia coli* O157 from minced beef. J. Food Protect., 60: 817-824.

Holland, JL; Louie, L; Simor, AE and Louie, M (2000). PCR detection of *Escherichia coli* O157:H7 directly from stools: evaluation of commercial extraction methods for purifying fecal DNA. J. Clin. Microbiol., 38: 4108-4113.

Jackson, SG; Goodbrand, RB; Jahnson, RP; Odorico, VG; Alives, D; Rahn, K; Wilson, JB; Welch, MK and Khakhiria, R (1998). *Escherichia coli O157:H7* diarrhea associated with well water and infected cattle on an Ontario farm. Epidemiol. Infect., 120: 17-20.

Johnsen, G; Wasteson, Y; Heir, E; Berget, OI and Herikstad, H (2001). *Escherichia coli* O157:H7 in faeces from cattle, sheep and pigs in the Southwest part of Norway during 1998 and 1999. Int. J. Food Microbiol., 65: 193-200.

Lahti, E; Keskimaki, M; Rantala, L; Hyvonen, P; Siitonen, A and Honkanen-Buzalski, B (2001). Occurrence of *Escherichia coli* O157 in Finnish cattle. Vet. Microbiol., 79: 239-251.

Nataro, JP and Kaper, JB (1998). Diarrheagenic *Escherichia coli*. Clin. Microbiol. Rev., 11: 142-201.

Nielsen, EM; Tegtmeier, C; Andersen, HJ; Gronbaek, C and Andersen, JS (2002). Influence of age, sex and herd characteristics on the occurrence of verocytotoxin producing *Escherichia coli* O157 in Danish dairy farms. Vet. Microbiol., 88: 245-257.

Oksuz, O; Arici, M; Kurultay, S and Gumus, T (2004). Incidence of *Escherichia coli* O157 in raw milk and white pickled cheese manufactured from raw milk in Turkey. Food Control. 15: 453-456.

Paton, AW and Paton, JC (1998). Detection and characterization shiga toxigenic *Escherichia coli* by using multiplex PCR assays for *stx*$_1$, *stx*$_2$, *eae*A, Enterohemorrhagic *E. coli hly*A, *rfb* 0111 and *rfb* O157. J. Clin. Microbiol., 36: 598-602.

Paton, AW; Paton, JC; Goldwater, PN and Manning, PA (1993). Direct detection of *Escherichia coli* shiga-like toxin genes in primary fecal cultures using the polymerase chain reaction. J. Clin. Microbiol., 31: 3063-3067.

Riley, LW; Remis, RS; Helgerson, SD; McGee, HB; Wells, JG; Davis, BR; Hebert, RJ; Olcott, ES; Johnson, LM; Hargrett, NT; Blake, PA and Cohen, ML (1983). Hemorrhagic colitis associated with a rare *Escherichia coli* serotype. New Engl. J. Med., 308: 681-685.

Sanderson, MW; Sargeant, JM and Nagaraja, TG (2005). Effect of pooling bovine fecal samples on the sensitivity of detection of *E. coli* O157:H7. Vet. Microbiol., 110: 125-130.

Whipp, SC; Rasmussen, MA and Cray, WC (1994). Animals as a source of *Escherichia coli* pathogenic for human beings. JAVMA., 204: 1168-1175.