Iranian Journal of Veterinary Research, Shiraz University, Vol. 12, No. 2, Ser. No. 35, 2011

Short Paper

Identification of shiga toxin producing *Escherichia coli* O157:H7 in raw cow milk samples from dairy farms in Mashhad using multiplex PCR assay

Brenjchi, M.[1]; Jamshidi, A.[2*]; Farzaneh, N.[3] and Bassami, M. R.[3]

[1]Graduated from Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran; [2]Department of Food Hygiene and Public Health, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran; [3]Department of Clinical Sciences, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran

[*]**Correspondence:** A. Jamshidi, Department of Food Hygiene and Public Health, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran. E-mail: ajamshid@ferdowsi.um.ac.ir

(Received 20 Feb 2010; revised version 17 Oct 2010; accepted 30 Oct 2010)

Summary

In this study 130 bulk tank milk samples which were delivered to the Pegah Pasturisation Factory in Mashhad were collected randomly during the summer months. Samples were firstly enriched in modified trypticase soy broth containing novobiocin, followed by plating onto sorbitol MacConkey agar supplemented with cefixime and potassium tellurite for isolation of *Escherichia coli* O157:H7. Consequently the suspected non-sorbitol fermenting (NSF) colonies were confirmed by biochemical tests as *Escherichia coli* and then were used for multiplex-PCR assay, using primers specific for O157 and H7 antigens genes and then primers specific for *stx1* and *stx2* genes. NSF *Escherichia coli* colonies were recovered from 8 samples, and in multiplex-PCR assay one sample (0.77%) was confirmed as *Escherichia coli* O157:H7. The second multiplex PCR assay showed that the isolate was harboring the *stx2* gene. The PCR assay used in this study may be a possible alternative to immunological assays which detect somatic and flagellar antigens. Besides, this procedure determines the potential of isolates for toxin production.

Key words: *Escherichia coli* O157:H7, Bulk Tank Milk, PCR

Introduction

Escherichia coli O157:H7 serotypes are identified as enterohemoragic *E. coli* and categorized in shiga-like toxin producing *Escherichia coli* (STEC) (Oksuz *et al*., 2004). It causes haemorrhagic colitis, hemolytic-ureamic syndrom (HUS) and thrombotic thrombocytopenic purpura (TTP) (Zhao *et al*., 1998).

Cattle, especially the young ones, have been implicated as a principal reservoir of *E. coli* O157:H7 (Zhao *et al*., 1995). Cattle frequently excrete the bacteria in their feces (Molina *et al*., 2003). Therefore, insufficient heat-treatment of raw milk forms a potential infection risk (Betts, 2000), and the processing conditions for different milk products are very important, for the risk of survival of the bacterium.

Numerous methods have been designed specifically for the isolation of this serotype in clinical, food and environmental specimens. Among different selective media, MacConkey agar containing sorbitol instead of lactose (SMAC), is most commonly used for isolation of *E. coli* O157:H7 (March and Ratnam, 1986), and non-sorbitol fermenting colonies are potentialy considered as *E. coli* O157:H7 (McDonough *et al*., 2000). Sorbitol-MacConkey supplemented with cefixime and tellurite (CT-SMAC) increases the sensitivity of this medium (Sanderson *et al*., 1995). Furthermore, other than the culture methods which are based on biochemical

characteristics of the bacteria, many other assays have been developed, including serological techniques, which use both polyclonal and monoclonal antibodies specific for the O and H antigens (De Boer and Heuvelink, 2000). Molecular approaches have also been practiced. In this regard, PCR assays based on the presence or absence of specific virulence genes such as the *stx1*, *stx2*, *eaeA*, *hlyA* and serogroup-specific genes encoding O-antigens or H-antigens in *E. coli* serogroups have been described (Gannon *et al*., 1997; Kimura *et al*., 2000; Philpott and Ebel, 2003).

The aim of the present study was the isolation of *E. coli* O157:H7 from bulk tank milk samples which were delivered to Pegah Pasturisation Factory in Mashhad, by conventional culture method and confirmation by a serogroup-specific multiplex-PCR assay and to determine the presence of *stx1* and *stx2* genes.

Materials and Methods

Sample collection

A total of 130 samples from bulk tank milk collected from different dairy herds in a Mashhad suburb and delivered to a pasturisation factory, were obtained randomly during the summer months. The samples were brought to the laboratory on crushed ice.

Culture conditions

Immediately in the laboratory, 25 ml of each sample was aseptically transfered to 225 ml of modified tryticase soy broth (mTSB, Himedia, Mumbai India) containing 20 mg L^{-1} novobiocin, followed by incubation at 37°C for 24 h. The enriched culture were plated onto sorbitol MacConkey agar supplemented with cefixime (0.05 mg L^{-1}) and potassium tellurite (2.5 mg L^{-1}) (CT-SMAC, Merck, Germany) and incubated at 37°C for 24 h. Five non-sorbitol fermenting (NSF) colonies from each CT-SMAC plate were selected and streaked onto plates containing eosin methylen blue agar (EMB) and were incubated at 37°C for 24 h. These isolates, with typical *E. coli* metallic sheen on EMB, were characterized by biochemical tests, including conventional indole production, methyl red, voges proskauer, citrate utilization and lysine decarboxylase tests.

Multiplex-PCR assay

NSF colonies on CT-SMAC that had been confirmed as *E. coli* were used for PCR assays. *Escherichia coli* O157:H7 (ATCC-35150) was used as a positive control and sterile distilled water as the negative control. A whole-cell suspension was prepared by suspending an NSF bacterial colony from CT-SMAC in sterile distilled water. The cell lysate was made by heating the suspension for 10 min in a boiling water bath. The lysate were spine for 5 min at 6000 rpm to pellet the cellular debris. A volume of 2 µl of the supernatant was used as template for amplification by m-PCR. The presence of *flic* H7 gene encoding the flagellar antigen H7 and *rfb* O157 gene, which endcodes the somatic antigen O157 (Desmarchelier *et al*., 1998; Philpott and Ebel, 2003) were examined. Colonies which were confirmed as *E. coli* O157:H7 were subjected to the second m-PCR assay using primers specific for *stx1* and *stx2* genes (Holland *et al*., 2000). Table 1 describes the oligonuclotide sequence of primers used in the m-PCR assays. Each m-PCR reaction was performed in a 25 µl amplification mixture consisting of 2.5 µl 10 X PCR buffer (500 mM KCl, 200 mM Tris HCl), 0.5 µl dNTPs (10 mM), 1 µl $MgCl_2$ (50 mM), 1.25 µl of each primer (0.5 µM), 0.2 µl of Taq DNA polymerase (5 unit/µl) and 2 µl of template. The thermocycler (Bio Rad, Hercules, CA) program was started with initial incubation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 60 sec, annealing at 52°C for 30 sec and elongation at 72°C for 60 sec, and final extention at 72°C for 10 min. The PCR products were separated by electrophoresis in 1.5% agarose gel at 100 V for 40 min in tris-acetate buffer, visualized by ethidium bromide staining, illuminated by UV-transilluminator and documented by a gel documentation apparatus. 100 bp DNA ladder was used as a marker for m-PCR assay. The expected size of products for *rfb* O157 and *flic* H7 genes amplification were 259 and 625 bp and for *stx1* and *stx2* genes were 614 and 779 bp, respectively.

Table 1: Target genes, primers sequences and expected sizes for *Escherichia coli* O157:H7

Target gene	Primer sequence	Size (bp)
rfb O157	F: 5′- CGG ACA TCC ATG TGA TAT GG -3′ R: 5′- TTG CCT ATG TAC AGC TAA TCC -3′	259
flic H7	F: 5′- GCG CTG TCG AGT TCT ATC GAG-3′ R: 5′- CAA CGG TGA CTT TAT CGC CAT TCC-3′	625
stx1	F: 5′- ACA CTG GAT GAT CTC AGT GG-3′ R: 5′- CTG AAT CCC CCT CCA TTA TG-3′	614
stx2	F: 5′- CCA TGA CAA CGG ACA GCA GTT-3′ R: 5′- CCT GTC AAC TGA GCA CTT TG-3′	779

Results

From 130 bulk tank milk samples, non-sorbitol fermenting (NSF) colonies were isolated from 8 samples after enrichment and selective plating, but one isolate was confirmed as *E. coli* by biochemical tests. In m-PCR assay, using specific primers for *rfb* O157 and *flic* H7 genes, the isolate was confirmed as *E. coli* O157:H7 (Fig. 1). The second m-PCR assay, using specific primers for *stx1* and *stx2* genes, showed that the isolate was harboring only the *stx2* gene (Fig. 2).

Discussion

In this study *E. coli* O157:H7 was isolated from 0.77% of bulk tank milk samples by conventional culture method and confirmed by multiplex-PCR assay. Our findings do not differ greatly from those reported abroad from raw cow milk. It was reported that 6% of raw cow milk samples examined in Egypt and 3% in Austria were contaminated with *E. coli* O157:H7 (Abdul-Raouf *et al*., 1996; Allerberger and Dierich, 1997), but Klie *et al*. (1997) found that only 0.3% of the milk samples were contaminated with this serotype in Germany. Similar studies on raw cow's milk performed in the UK (Scotland) analyzing 500 samples (Coia *et al*., 2001) and in the Netherlands analyzing 1011 samples (Heuvelink *et al*., 1998a) resulted in no *E. coli* O157:H7 isolation. Although according to our finding the incidence of *E. coli* O157:H7 in bulk tank milk is low, considering the low infective dose, the presence of this pathogen in raw cow milk is important in this area of Iran.

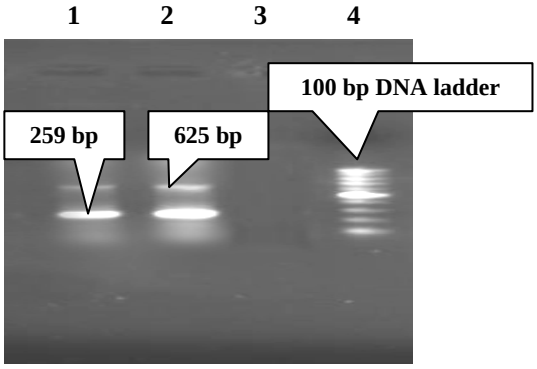

Fig. 1: Results of the m-PCR assay, amplifying 259 base pair segment of *rfb* O157 and 625 bp of *flic* H7 genes of *Escherichia coli* 157:H7. 1: Positive sample, 2: Positive control, 3: Negative control, and 4: 100 bp marker

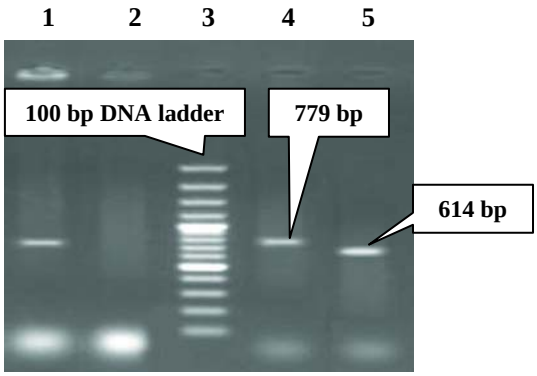

Fig. 2: Results of the m-PCR assay, amplifying 614 bp segment of *stx1* and 779 base pair of *stx2* genes of *Escherichia coli* O157:H7. 2: Negative control, 3:100 bp marker, 5: Positive control for *stx1-4*: Positive control for *stx2-1*: positive sample

The samples were collected during the summer months. Seasonal distribution of *E. coli* O157:H7 have been reported previously (Cagney *et al*., 2004), with the highest prevalence in summer and the lowest in winter, so it is possible that the contamination rate became even lower than 0.77% in other seasons.

The prevalence rates of pathogens in bulk tank milk could be influenced by

several other factors such as geographical area, farm size, number of animals on the farm, hygiene, and farm management practices (Rohrbach *et al*., 1992).

Modified tryticase soy broth was used in the enrichment stage. It has been proposed that the enrichment before plating on selective agar may increase the sensitivity of *E. coli* O157:H7 isolation compared to direct plating of samples on selective agar (Sanderson *et al*., 1995).

After the enrichment stage CT-SMAC agar was used for selective plating, because it has been found that the CT-SMAC agar medium yields the best results for selective cultivation of *E. coli* O157:H7 (De Boer and Heuvelink, 2000). Although using rainbow agar (a new chromogenic medium for the detection of *E. coli* O157:H7) has been found to be more sensitive than CT-SMAC, the difference was not significant (Tutenel *et al*., 2003).

m-PCR assay was used for confirming the non sorbitol fermenting colonies as *E. coli* O157:H7. Several researchers have reported multiplex PCR as a reliable identification method for *E. coli* O157:H7 (Gannon *et al*., 1997).

We used primers specific for flagellar and somatic antigens genes in the first multiplex-PCR assay. These antigens could be detected by immunological assays as well, but the main advantage of the employed m-PCR method is its ability to detect rough isolates or the isolates having a masked O antigen (Desmarchelier *et al*., 1998). The method developed in this study can also detect O157 H^- serotype of *E. coli*, although this serotype was not detected in our study.

In the second multiplex-PCR assay the presence of the main virulence genes (*stx1* and *stx2*), which have been widely used by other researchers (Holland *et al*., 2000; Keen and Elder, 2002) were investigated. According to our results, *stx2* was detected in isolated *E. coli* O157:H7, but *stx1* was not detected. It has been reported that the *stx2* gene was more common than the *stx1* in most of the studies performed in the USA, Japan and European countries (Zhao *et al*., 1995; Johnsen *et al*., 2001; Keen and Elder, 2002).

These two multiplex PCR assays are suitable methods for the rapid identification of *E. coli* O157:H7 to species level and determining their virulence genes, as it could speed up and simplify the identification procedures that could be completed in 1 working day.

References

Abdul-Raouf, UM; Ammar, MS and Beuchat, LR (1996). Isolation of *Escherichia coli* O157:H7 from some Egyptian foods. Int. J. Food Microbiol., 29: 423-426.

Allerberger, F and Dierich, MP (1997). Enterohemorrhagic *Escherichia coli* in Austria. VTEC'97, abstract V37/I p.4 3rd international symposium and workshop on shiga toxin (Verocytotoxin)-producing *Escherichia coli* infections. Baltimore, MD, USA. Lois Joy Galler Foundation for HUS, Melville, NY, USA.

Betts, GD (2000). Controlling *E. coli* O157:H7. Nutr. Food Sci., 30: 183-186.

Cagney, C; Crowley, H; Duffya, G; Sheridan, JJ; O'Brien, S; Carney, E; Anderson, W; McDowell, DA; Blair, IS and Bishop, RH (2004). Prevalence and numbers of *Escherichia coli* O157:H7 in minced beef and beef burgers from butcher shops and supermarkets in the Republic of Ireland. Food Microbiol., 21: 203-212.

Coia, JE; Johnston, Y; Steers, NJ and Hanson, MF (2001). A survey of the prevalence of *Escherichia coli* O157 in raw meats, raw cow's milk and raw-milk cheeses in south-east Scotland. Int. J. Food Microbiol., 66: 63-69.

De Boer, E and Heuvelink, AE (2000). Methods for detection and isolation of shiga toxin-producing *Escherichia coli*. Symp. Ser. Soc. Appl. Microbiol., 29: 133-143.

Desmarchelier, PM; Bilge, SS; Fegan, N; Mills, L; Vary, JC and Tarr, PI (1998). A PCR specific for *Escherichia coli* O157:H7 based on *rfb* locus encoding *O157* lipopolysaccharide. J. Clin. Microbiol., 36: 1801-1804.

Gannon, VP; D'Souza, S; Graham, T; King, RK; Rahn, K and Read, S (1997). Use of the flagellar H7 gene as a target in multiplex PCR assays and improved specificity in identification of enterohemorrhagic *Escherichia coli* strains. J. Clin. Microbiol., 35: 656-662.

Heuvelink, AE; Bleumink, B; van den Biggelaar, FL; Te Giffel, MC; Beumer, RR and de Boer, E (1998a). Occurrence and survival of verocytotoxin-producing *Escherichia coli*

O157:H7 in raw cow's milk in the Netherlands. J. Food Prot., 61: 1597-1601.

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.

Johnsen, G; Yngvild, W; 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.

Keen, EJ and Elder, RO (2002). Isolation of shiga-toxigenic *Escherichia coli* O157 from hide surface and the oral cavity of finished beef feedlot cattle. JAVMA. 220: 756-763.

Kimura, R; Mandrell, RE; Galland, JC; Hyatt, D and Riley, LW (2000). Restriction-site-specific PCR as a rapid test to detect enterohemorrhagic *Escherichia coli* O157:H7 strains in environmental samples. Appl. Environ. Microbiol., 66: 2513-2519.

Klie, H; Timm, M; Richter, H; Gallien, P; Perlberg, KW and Steinruck, H (1997). Detection and occurrence of verotoxin-forming and/or shigatoxin producing *Eschericia coli* (VTEC and/or STEC) in milk. Berl. Munch. Tierarztl. Wochenschr., 110: 337-341.

March, SB and Ratnam, S (1986). Sorbitol-MacConkey medium for detection of *Escherichia coli* O157:H7 associated with hemorrhagic colitis. J. Clin. Microbiol., 23: 869-872.

McDonough, PL; Rossiter, CA; Rebhun, RB; Stehman, SM; Lein, DH and Shin, SJ (2000). Prevalence of *Escherichia coli* O157:H7 from cull dairy cows in New York state and comparison of culture methods used during preharvest food safety investigations. J. Clin. Microbiol., 38: 318-322.

Molina, PM; Parma, AE and Sanz, ME (2003). Survival in acidic and alcoholic medium of shiga toxin-producing *Escherichia coli* O157:H7 and non-O157:H7 isolates in Argentina. BMC Microbiol., 13: 3-17.

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.

Philpott, D and Ebel, F (2003). *E. coli: shigatoxin methods and protocols*. 1st Edn., Totowa, New Jersey, Humana Press Inc., PP: 9-45.

Rohrbach, RW; Draughon, FA; Davidson, PM and Oliver, SP (1992). Prevalence of *Listeria monocytogenes*, *Campylobacter jejuni*, *Yersinia enterocolitica* and *Salmonella* in bulk tank milk: risk factors and risk of human exposure. J. Food Prot., 55: 93-97.

Sanderson, MW; Gay, JM; Hancock, DD; Gay, CC; Fox, LK and Besser, TE (1995). Sensitivity of bacteriologic culture for detection of *Escherichia coli* O157:H7 in bovine feces. J. Clin. Microbiol., 33: 2616-2619.

Tutenel, AV; Pierard, D; Vandekerchove, D; Van Hoof, J and De Zutter, L (2003). Sensitivity of methods for the isolation of *Escherichia coli* O157 from naturally infected bovine feces. Vet. Microbiol., 94: 341-346.

Zhao, T; Doyle, MP; Harmon, BG; Brown, CA; Eric Mueller, PO and Parks, AH (1998). Reduction of carriage of Enterohemorrhagic *Escherichia coli* O157:H7 in cattle by inoculation with probiotic bacteria. J. Clin. Microbiol., 36: 641-647.

Zhao, T; Doyle, MP; Share, J and Garber, L (1995). Prevalence of enterohemorrhagic *Escherichia coli* O157:H7 in a survey of dairy herds. Appl. Environ. Microbiol., 61: 1290-1293.