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Full Length Research Paper

Direct detection of *Escherichia coli* O_{157} and its major virulence factor genes in animal faeces at slaughter using multiplex polymerase chain reaction (PCR)

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Although more than 100 serotypes of shigatoxigenic *Escherichia coli* (STEC) have been implicated in cases of human diseases, *E. coli* O_{157} is the most common serogroup connected with sporadic cases and large outbreaks of diseases in many countries. Rapid and sensitive identification of this dangerous pathogen is important for patient management and for prompt epidemiological investigations. PCR has become a very rapid and reliable tool for the molecular diagnosis of *E. coli* O_{157}. PCR assays are usually aimed at detecting the shiga toxins, the intimin protein and enterohaemolysin. In the present study, a mPCR-based protocol is described as that which uses one primer set to detect the gene responsible for the production of the O- antigen synthesis (*rfb* O_{157}) and four primer set to detect the major virulence factor genes including the Shigatoxin type 1 and 2 (*stx*$_1$ and *stx*$_2$), intimin (*eaeA*) and enterohemolysin (EHEC *hlyA*) directly from 190 samples of animal faeces at the time of slaughter after overnight incubation of stool specimens in BPW. In this research, we use one primer set for detection of the gene responsible for the production of the O- antigen synthesis (*rfb* O_{157}) and four primer set for detection of the Shigatoxin type 1 and 2 (*stx*$_1$ and *stx*$_2$), intimin (*eaeA*) and enterohemolysin (EHEC *hlyA*) producing genes directly from 190 samples of animal faeces at the time of slaughter after overnight incubation of stool specimens in BPW. This study has established the presence of rather high prevalence of *E. coli* O_{157}-positive animals at abattoirs (These consisted of 4.2% of cattle and 2.1% of sheep), providing an increased risk of transmission of *E. coli* O_{157} to the food chain and contamination of human.

Key words: Shigatoxigenic *Escherichia coli* (STEC), *E. coli* $O_{157,}$ shiga toxins, intimin, enterohaemolysin, mPCR.

INTRODUCTION

Escherichia coli is generally recognized as an enteric commensal, but the species also includes a significant pathogenic group frequently associated with severe human gastrointestinal problems that include bloody diarrhea, hemorrhagic colitis (HC), and the life-threatening hemolytic uremic syndrome (Griffin and Tauxe, 1991; Tarr, 1995). Although more than 100 serotypes of shigatoxigenic *E. coli* (STEC) have been implicated in cases of human diseases (Paton and Paton, 1998; Meng et al., 2007), *E. coli* O_{157} that was first recognized as a food-borne pathogen in 1982 during an outbreak that was traced to contaminated hamburgers (Riley and Remis, 1983), is the most common serogroup connected with sporadic cases and large outbreaks of diseases in many countries (Griffin and Tauxe, 1991; Armstrong et al., 1996).

Cattle and other ruminants have been established as major natural reservoirs for *E. coli* O_{157} and play an important role in the epidemiology of human infections

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(Rassmussen et al., 1993). So, consumption of contaminated food of animal origin can be the main rout of transmission of *E. coli* O_{157} to human and shedding of the pathogen by animals can pose elevated risk of contaminating the food chain if presented to slaughter. Therefore, rapid and sensitive identification of this dangerous pathogen is important for prompt epidemiological investigations.

The development of molecular methodologies to detect STEC in clinical and environmental samples has led to more precise and rapid determination of this public health risk. PCR has become a very rapid and reliable tool for the molecular biology-based diagnosis of a variety of microorganisms from microbial cultures and directly from clinical samples (Hu et al., 1999; Holland et al., 2000). PCR assays are usually aimed at detecting the shiga toxins, encoded by bacteriophage genes, the intimin protein that is involved in the intimate adhesion of bacteria to enterocytes and the plasmid-encoded enterohaemolysin (Jackson et al., 1987; Beutin et al., 1989; Paton and Paton, 1998; Law, 2000; Pass et al., 2000; Osek, 2003).

In the present study, a mPCR-based protocol is described as that which uses one primer set to detect the gene responsible for the production of the O- antigen synthesis (*rfb* O_{157}) and four primer set to detect the major virulence factor genes including the Shigatoxin type 1 and 2 (stx_1 and stx_2), intimin (*eaeA*) and enterohemolysin (EHEC *hlyA*) directly from animal faeces at the time of slaughter. The prevalence of *E. coli* O_{157} in slaughtered animal faeces was also investigated.

MATERIALS AND METHODS

Sample collection and preparation

Weekly samples (totally 190 stool specimens consist of 120 samples of cattle faeces and 70 samples of sheep faeces) were collected from Tabriz slaughterhouse between June and August 2010. Approximately 50 g of fecal material was taken from the rectum of each animal after eviscerating of carcasses. This was done on the process line of the factory by manually milking of the fecal contents. Samples were collected in sterile plastic bags, stored in a cool box, and transported to the laboratory. All samples were stored at 4°C and processed within 8 h of collection. Each fecal sample (10 g) was homogenized with 90 ml of buffered peptone water and was incubated in 37°C overnight. Each 10 ml volume of the mixture of each sample was filtered and transferred to a clean tube, and tubes were centrifuged at 1500 rpm for 3 min. The resultant pellet was collected and resuspended in equal volume of BPW in another tube.

DNA extraction

DNA was extracted by centrifugation of 1.5 ml of resuspension solution in Eppendrof tube at 10000 rpm for 5 min and removal of the supernatant. A volume of 800 µl of lyses buffer solution (5 mol NaCl, 100 mM Tris-base, 20 mM EDTA-Na2 and CTAB 20%) were added and the samples were vortexed. The tubes were incubated at 65°C for 15 min and were centrifuged again at 12000 rpm for 10 min. Equal volume of chloroform – isoamylalcohol (24:1) were added to the supernatants and the tubes were centrifuged at 12000 rpm for 10 min. The supernatants were then transferred to new tubes and after adding equal volume of –20°C isopropanol and keeping at -20°C for 30 min, the samples were centrifuged at 12000 rpm for 10 min and the supernatants were discarded. Finally 250 µl of 4°C ethanol (70%) were added and the samples centrifuged at 10000 rpm for 5 min. The supernatants were discarded and the pellets were dried at room temperature and were dissolved in 50 µl of sterile distilled water and were reserved as the PCR template. DNA standards were extracted from *E. coli* O_{157}:H_7 (ATCC 25922) known to contain the relevant genes (Atashpaz et al., 2010).

PCR tests

The oligonucleotide primers (*rfb* O_{157}, stx_1, stx_2, *eaeA* and EHEC *hlyA*) used in this study ranging from 20 to 22 primers were selected from the published sequences and were chosen to have adjacent annealing temperatures and minimal interactions and resulted in different-sized products distinguishable in agarose gels (Gannon et al., 1992; 1997; Paton and Paton, 1998). Target primers for amplifying segments of genes are listed in Table 1.

Initially, 0.4 µM of each set of primers and 50 ng of extracted DNA were used but this approach resulted in uneven intensivity of the amplified products. Overcoming this problem required changing the proportions of the primers in the reaction mixture. So, mPCR was performed with a reaction mixture containing 30 ng of extracted DNA of each stool specimens, 12.5 µl 2X PCR primer master kit (Cinagene, Iran 616301394), 0.15 µM of each primers and distilled water to the final volume of 25 µl.

The reactions were carried out with amplification thermal cycler (ASTEC, PC-818A). The procedure consisted of initial denaturation at 94°C for 4 min, followed by 35 cycles of template denaturation at 94°C for 50 s, primer annealing at 57°C for 50 s and extension at 72°C for 1 min. The final cycle was followed by incubation of the reaction mixture for 10 min at 72°C.

Reaction products were separated by agarose gel electrophoresis by adding 1 µl of 6X loading dye to reaction mixture and loading 6 µl onto a 1.5% agarose gel (Invitrogen, G501802). The buffer in the electrophoresis chamber and in the agarose gel was TAE 1X and contained 2 µl Sibergreen (Nanobiotech, M1742-1) per 100 ml gel. 80 volts were applied across the gel. Finally the gel was visualized by exposing the gel to UV light and was photographed. DNA standard molecular weight size marker (100 bp – Fermentas, SN 0623) was included in each agarose gel electrophoresis run.

RESULTS

Multiplex PCR assays were considered positive for *E. coli* O_{157} when *rfb*O_{157} gene was amplified. *E. coli* O_{157} carriage in the faeces of individual animals tested was 6.3%. These consisted of 4.2% cattle and 2.1% sheep stool samples.

Figure 1 shows amplification of the target gene segments in *E. coli* O_{157}-positive samples. Among the *E. coli* O_{157} -positive bacteria analyzed in the present study, two isolates (both from cattle) had all targeted genes as standard strain and one isolate from cattle faeces had the stx_1 gene together with the EHEC *hlyA*. Five isolates including three samples from cattle and two samples from sheep had the stx_1 gene and one isolate from cattle faeces had just EHEC *hlyA* gene. Three samples had no

Table 1. Primer sequences and predicted length of PCR amplification product.

Primer	Primer sequence (5′-3′)	Size	Reference
rfb O_{157}- F *rfb* O_{157}- R	CGG ACA TCC ATG TGA TAT GG TTG CCT ATG TAC AGC TAA TCC	259	Paton and Paton (1998)
stx_1- F stx_1- R	ACA CTG GAT GAT CTC AGT GG CTG AAT CCC CCT CCA TTA TG	614	Gannon et al. (1992)
stx_2- F stx_2- R	CCA TGA CAA CGG ACA GCA GTT CCT GTC AAC TGA GCA CTT TG	779	Gannon et al. (1997)
eaeA- F *eaeA*- R	GTG GCG AAT ACT GGC GAG ACT CCC CAT TCT TTT TCA CCG TCG	890	Gannon et al. (1997)
EHEC *hlyA*- F EHEC *hlyA*-R	GCA TCA TCA AGC GTA CGT TCC AAT GAG CCA AGC TGG TTA AGC T	534	Paton and Paton (1998)

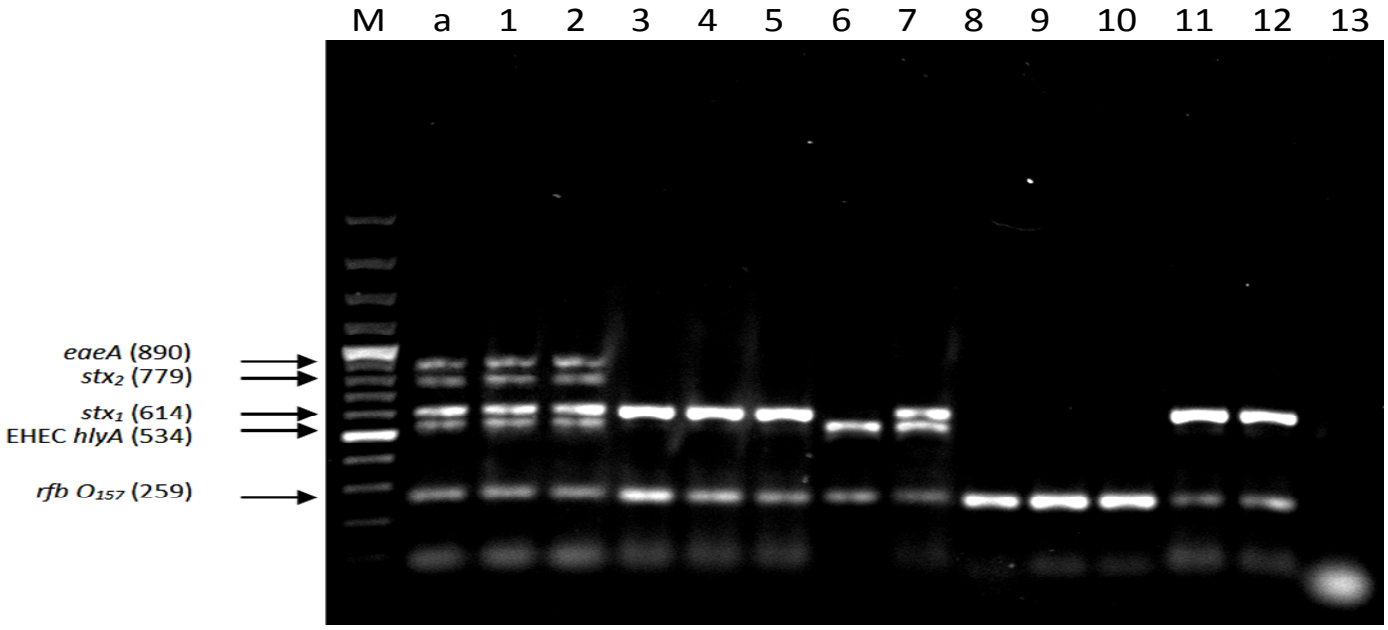

Figure 1. Multiplex PCR assay with *rfb* O_{157}, stx_1, stx_2, *eaeA* and EHEC *hlyA* primer pairs. Lanes M: 100 bp DNA size marker (fermentase, SN 0623), Lane a: *E. coli* O_{157}:H_7 (ATCC 25922), Lanes 1 to 8: Stool specimens from cattle that were *rfbO*$_{157}$ positive, Lanes 9 to 12: Stool specimens from sheep that were *rfbO*$_{157}$ positive and Lane 13: PCR assay control (no template DNA).

virulence factor gene. As shown in Figure 1, majority of *E. coli* O_{157} organisms (67%) isolated belonged to shigatoxin-producing *E. coli* (STEC) family recognized as serious threat to public health.

DISCUSSION

Various PCR-based strategies to look for *E. coli* O_{157} and other STEC serogroups have been developed. Some of the PCR tests for the detection of STEC use single pair Zof primers which are able to amplify a single gene related to one pathogen or single virulence factor gene of pathogens (Lin et al., 1993; Louie et al., 1994; Thomas et al., 1994; Tornieporth et al., 1995), whereas others combine different primer pairs in one reaction tube (mPCR). Multiplex PCRs which simultaneously amplify several STEC-associated genes have recently been described. Fagan et al. (1999) developed PCR assay that was able to identify stx_1 and stx_2, intimin (*eaeA*), and enterohemorrhagic *E. coli* Hemolysin (EHEC *hlyA*) genes in animal faeces or Holland et al. (2000) described a PCR

method for detecting STEC strains by amplifying the *stx*$_1$, *stx*$_2$ and *eaeA*O$_{157}$ genes. However, none of these methods was used for the direct detection of *E. coli* O$_{157}$genes targeted in present research, from fecal samples in a single reaction.

The mPCR-based approach described allowed for successful amplification of *rfbO*$_{157}$, *stx*$_1$, *stx*$_2$, *eaeA* and EHEC *hlyA* sequences in some isolates as in control strain, so to our knowledge, this report illustrates a useful diagnostic method for the identification of *E. coli* O$_{157}$ and its major virulence factor genes directly from broth cultures on animal faeces. 6.3% of examined animal were found to carry *E. coli* O$_{157}$ and the majority of *E. coli* O$_{157}$ organisms (75%) isolated were potentially pathogenic to humans having targeted virulence factor genes. The study was performed in the summer months, which is previously defined as the period of high prevalence (Hancock et al., 2001; Meng et al., 2007). Other possible reason for high number of positive cases could be that the animals may have shed increasing loads of *E. coli* O$_{157}$ from stress due to transport prior to slaughter (McCluskey et al., 1999). Also, the sensitivity of test was high because larger sample volumes were assayed in the present project (10 g compared to 1 g) or because, we used broth enrichment of stool specimens before PCR. Although direct extracts of faeces can be used as templates for PCR, the best results are usually obtained by testing extracts of primary broth cultures (Stacy-Phipps et al., 1995; Lantz et al., 1997). Broth enrichment serves two purposes: inhibitors in the sample are diluted, and bacterial growth increases the number of copies of the target sequence.

This study has established the presence of rather high prevalence of *E. coli* O$_{157}$-positive animals at abattoir that is a major link in the transmission of *E. coli* O$_{157}$ to the food chain by cross-contamination of the carcass and ground beef with faeces (Lantz et al., 1997; Richards et al., 1998; Slutsker et al., 1998) and the return of waste to the fields and to the crops (Lau and Ingham, 2001; Duffy, 2003; Avery et al., 2005; Fremaux et al., 2007; Khandaghi et al., 2010). So the need for suitable control measures for such animals cannot be underestimated. Further work is required to understand the range of existing concentrations of this group of pathogens because the presence of high-shedding animals at abattoirs highlights the risk of contamination to both the food chain and the environment.

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REFRENCES

Armstrong GL, Hollingsworth J, Morris JG (1996). Emerging foodborne pathogens: *Escherichia coli* O157:H7 as a model of entry of a new pathogen into the food supply of the developed world. Epidemiol. Rev., 18: 29 –51.

Atashpaz S, Khani S, Barzegari A, Barar J, Vahed SZ, Azarbaijani R, Omidi Y (2010) . A robust universal method for extraction of genomic DNA from bacterial species. Mikrobiologiia., 79(4):562-566.

Avery LM, killham K, Jones DL (2005). Survival of *E. coli* O157:H7 in organic wastes destined for land application. J. Appl. Microbiol., 98: 814-822.

Beutin L, Montenegro MA, Orskov I, Orskov F, Prada J, Zimmermann S, Stephan R (1989). Close association of verotoxin (Shiga-like toxin) production with enterohemolysin production in strains of *Escherichia coli*. J. Clin. Microbiol., 27: 2559–2564.

Duffy G (2003). Verotoxigenic *E. coli* in animal faeces, manures and slurries. J. Appl. Microbiol., 94: 945-1035.

Fagan PK, Hornitzky MA, Bettelheim KA, Djordjevic SP (1999). Detection of Shiga-Like Toxin (*stx1* and *stx2*), Intimin (*eaeA*), and Enterohemorrhagic *Escherichia coli* (EHEC) Hemolysin (EHEC *hlyA*) Genes in Animal Faeces by Multiplex PCR. Appl. Environ. Microbiol., 65: 868–872.

Fremaux B, Delignette-Muller ML, Prigent-combaret C, Gleizal A, vernozy-Rozand C (2007). Growth and Survival of non-O157: H7 shigatoxin- producing *E. coli* in cow manure. J. Appl. Microbiol., 102: 89-99.

Gannon VPJ, Dsouza S, Graham T, King RK, Rohn K, Read S (1997). Use of the flagellar H7 gene as target in multiplex PCR assays and improved specificity in identification of entrohemorrhagic *Escherichia coli* strains. J. Clin. Microbiol., 35: 656-622.

Gannon VPJ, King RK, Kim JY, Thomas EJG (1992). Rapid and sensitive method for detection of Shiga-like toxin-producing *Escherichia coli* in ground beef using the polymerase chain reaction. Appl. Environ. Microbiol., 58: 3809–3815.

Griffin PM, Tauxe RV (1991). The epidemiology of infections caused by *Escherichia coli* O157:H7, other enterohemorrhagic *E. coli*, and the associated hemolytic uremic syndrome. Epidemiol. Rev., 13: 60–98.

Hancock D, Besser T, Lejeune J, Davis M, Rice D (2001). The control of VTEC in the animal reservoir. Int. J. Food Microbiol., 66: 71–78.

Holland JL, Louie L, Simora E, Louiei 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.

Hu Y, Zhang Q, Meitzler JC (1999). Rapid and specific detection of *Escherichia coli* O157:H7 in bovine faeces by a multiplex PCR. J. Appl. Microbiol., 87: 867–876.

Jackson MP, Neill RJ, OBrien AD, Holmes RK, Newland JW (1987). Nucleotide sequence analysis and comparison of the structural genes for Shiga-like toxin I and Shiga-like toxin II encoded by bacteriophages from *Escherichia coli.* FEMS Microbiol. Lett., 44: 109–114.

Khandaghi J, Razavilar V, Barzgari A (2010). Isolation of *Escherichia coli* O157:H7 from manure fertilized farms and raw vegetables grown on it, in Tabriz city in Iran. Afr. J. Microbiol. Res., 9: 891-895.

Lantz PG, Matsson M, Wadstrom T, Radstrom P (1997). Removal of PCR inhibitors from human fecal samples through the use of an aqueous two-phase system for sample preparation prior to PCR. J. Microbiol. Meth., 28: 159–167.

Lau MM, Ingham SC (2001). survival of fecal indicator bacteria in bovine manure incorporated into soil. Lett. Appl. Microbiol., 33: 131-136.

Law D (2000). (A review) virulence factors of *Escherichia coli* O157. J. Appl. Microbiol., 88: 729-745.

Lin Z, Kurazono H, Yamasaki S, Takeda Y (1993). Detection of various variant verotoxin genes in *Escherichia coli* by polymerase chain reaction. Microbiol. Immunol., 37: 543–548.

Louie M, de Azavedo J, Clarke R, Borczyk A, Lior H, Richter M, Brunton J (1994). Sequence heterogeneity of the *eae* gene and detection of verotoxin-producing *Escherichia coli* using serotype-specific primers. Epidemiol. Infect., 112: 449–461.

McCluskey BJ, Rice DH, Hancock D, Hovde CJ, Besser TE, Gray S, Johnson RP (1999). Prevalence of *Escherichia coli* O157 and other Shiga-toxin-producing *E. coli* in lambs at slaughter. J. Vet. Diagn. Investig., 11: 563–565.

Meng J, Doyle MP, Zhao T, Zhao S (2007). Entrohemorrhagic

Escherichia coli. In: Doyle M.P and Beuchat, L.R, food microbiology: Fundamentals and frontiers. 3rd Ed. ASM press, Washigton D.C., pp. 249-268.

Osek J (2003). Development of a multiplex PCR approach for the identification of Shiga toxin-producing *Escherichia coli* strains and their major virulence factor genes. J. Appl. Microbiol., 95: 1217–1225.

Pass MA, Odedra R, Batt RM (2000). Multiplex PCRs for Identification of *Escherichia coli* Virulence Genes. J. Clin. Microbiol., 38: 2001–2004.

Paton AW, Paton JC (1998). Detection and Characterization of Shigatoxigenic *Escherichia coli* by Using Multiplex PCR Assays for *stx*1, *stx*2, *eaeA*, Enterohemorrhagic *E. coli hlyA*, *rfb*O111, and *rfb*O157. J. Clin. Microbiol., 36: 598–602.

Paton JC, Paton AW (1998). Pathogenesis and diagnosis of Shiga toxin-producing *Escherichia coli* infections. Clin. Microbiol. Rev., 11: 450–479.

Rassmussen MA, Cray WC, Casey TA, Whip SC (1993). Rumen content as a reservoir of enterohemorrhagic *E. coli.* FEMS Microbiol. Lett., 114: 79–84.

Richards MS, Corkish JD, Sayers AR, McLaren IM, Evans SJ, Wray C (1998). Studies of the presence of verocytotoxic *Escherichia coli* O157 in bovine faeces submitted for diagnostic purposes in England and Wales and on beef carcasses in abattoirs in the United Kingdom. Epidemiol. Infect., 120: 187–192.

Riley LW, Remis RS (1983). Hemorrhagic colitis associated with a rare *E. coli* serotype. N. Engl. J. Med., 308: 681–685.

Stacy-Phipps S, Mecca JJ, Weiss JB (1995). Multiplex PCR assay and simple preparation method for stool specimens detect enterotoxigenic *Escherichia coli* DNA during course of infection. J. Clin. Microbiol., 33: 1054–1059.

Slutsker L, Ries AA, Maloney K, Wells JG, Greene KD, Griffin PM (1998). The *E. coli* O157 Study Group A nationwide case-control study of *Escherichia coli* O157:H7 infection in the United States. J. Infect. Dis., 177: 962–966.

Tarr PI (1995). *Escherichia coli* O157:H7: clinical, diagnostic, and epidemiological aspects of human infection. Clin. Infect. Dis., 20: 1–8.

Thomas A, Jiggle B, Smith HR, Rowe B (1994). The detection of Verocytotoxin-producing *Escherichia coli* and *Shigella dysenteriae* type 1 in fecal specimens using polymerase chain reaction gene amplification. Lett. Appl. Microbiol., 19: 406–409.

Tornieporth NG, John J, Salgado K, de Jesus P, Latham E, Melo MC, Gunzburg ST, Riley LW (1995). Differentiation of pathogenic *Escherichia coli* strains in Brazilian children by PCR. J. Clin. Microbiol., 33: 1371–1374.