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Development of a multiplex-PCR for the rapid detection of *Escherichia coli* O157 : H7 from raw beef

Suk-chan Jung, Byeong-yeal Jung, Jang-won Yoon*, Yun-sang Cho, Jong-yeom Kim, Yong-ho Park*

Department of Bacteriology, National Veterinary Research Institute
*Department of Veterinary Medicine, Seoul National University**
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쇠고기중 *Escherichia coli* O157 : H7 신속검출을 위한 multiplex-PCR기법 개발

정석찬 · 정병열 · 윤장원* · 조윤상 · 김종염 · 박용호*

수의과학연구소 세균과
서울대학교 수의과대학*
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초 록 : *Esherichia coli* O157 : H7의 *slt* I, *slt* II, *uid* A 및 *eae*A 4종 유전자를 동시에 검출하기 위한 multiplex PCR 기법을 확립하고 쇠고기중 직접 *E coli* O157 : H7 검출시험을 실시하였다. 4 set의 primers를 이용한 multiplex PCR 기법으로 31종의 장내세균에 대한 특이성을 조사한 결과 *E coli* O157 : H7에서 1,087bp (*eae* A), 584bp (*slt* II), 348bp (*slt* I) 또는 252bp (*uid* A)크기의 DNA를 동시에 특이적으로 검출할 수 있었다. *E coli* O157 : H7 15주는 모두 *uid* A 및 *eae* A 유전자가 동시에 검출되었고, 다른 장내세균에서는 검출되지 않았다. *slt* I 또는 *slt* II 유전자를 가지고 있는 *E coli* 표준균주 24종을 이용하여 multiplex PCR 기법과 Vero cell cytotoxicity assay을 비교검사한 결과 베로톡신 산생능과 PCR법의 결과는 일치하였다. mutiplex PCR 기법의 쇠고기중 검출한계는 modified EC(mEC)에서 증균없이는 *E coli* O157 : H7균 10^4cells/g 이상에서 검출이 가능하였으나 mEC에 1차 증균후 modified TSB 증균하였을 경우에는 10cells/g이하까지도 검출이 가능하였다. 개발된 multiplex PCR 기법을 쇠고기 40종에 직접 적용한 결과 *E coli* O157 : H7은 검출되지 않았으나 *slt* I 및 *slt* II유전자를 가지고 있는 *E coli* 4종이 검출되었으며, 이들의 혈청형은 O6, O112, O115 및 O139 였다. 이 연구에서 개발된 multiplex PCR은 쇠고기중 *E coli* O157 : H7을 신속하고 특이적으로 검출하는데 사용할 수 있을 것으로 사료된다.

Key words : *E coli* O157:H7, multiplex PCR, *eae* A, *uid* A, *slt* I, *slt* II, raw beef.

Address reprint requests to Dr. Suk-chan Jung, Department of Bacteriology and Immunology, National Veterinary Research Institute, #480 Anyang 6-dong Anyang, Kyunggi, Korea 430-016.

Introduction

Escherichia coli O157 : H7 was first recognized as a major food-borne pathogen following two outbreaks in the United States in 1982 which were epidemiologically linked to the consumption of undercooked hamburgers[1]. After those accidents, several outbreaks of *E coli* O157 : H7 have been reported in the worldwide[2,3]. Infected individuals with the bacteria result in hemorrhagic colitis, hemolytic uremic syndrome and thrombotic thrombocytopenic purpura as clinical signs[1,3]. Unlike other serotypes of *E coli*, *E coli* O157 : H7 do not ferment sorbitol and is negative in β-D-glucuronidase (GUD) activity with the 4-methylumbelliferone β-D-glucuronide (MUG) assay[4,5]. Although selective media, sorbitol MacConkey agar and MUG medium have been used to identify *E coli* O157 : H7 from meats, confirmation has been required with antisera to O157 and H7 because of the presence of those properties in other bacteria[6]. Rapid and accurate methods for the detection of food-borne microbial pathogens have been required in public awareness. However, the standard microbiological techniques have several disadvantages such as time-consuming and laborious. Also, the methods have difficulties in isolation of the pathogenic bacteria due to overgrowth of competing microflora, limited selectivity, and false reactions[7-9].

Therefore, alternative methods such as immunoassays[10,11], PCR[7,12], and DNA hybridization[13-15] have been developed to solve the problems by using difference of culture properties in sorbitol or MUG reactions with the *E coli* strains. More than one genes were simultaneously amplified in one PCR reaction using multiple pairs of designed primers[16]. Especially, the method could be applicable to food industry by the increased specificity and capability to rapidly distinguish between nonpathogenic and pathogenic species.

Virulence factors of the *E coli* O157 : H7 are Shiga-like toxins (SLT, or verotoxins) produced from the bacteria and a 97kDa outer membrane protein, called intimin, to adhere on the intestinal mucosa[3,17]. The protein is produced from *eae*A gene and associated with an attaching and effacing (AE) lesion. The *eae* gene was common among clinical Shiga-like toxins producing *E coli* (SLTEC) isolates in humans while significantly less common among SLTEC isolates in healthy cattle. The result suggested that *eae* gene could be a better predictor for the pathogenic potential of SLTEC than the *slt* genes in human[12,15].

Recently, a multiplex PCR assay was developed by simultaneous amplification of the *slt* I, *slt* II and *uid* A genes of *E coli* O157 : H7. Even though *E coli* O157 : H7 carries the *uid* A gene, they can not express GUD activity because of point mutation(T to G) at position 92 of the gene[13]. Thus, the conserved base change in *uid* A allele might be a powerful marker for the identification of *E coli* O157 : H7[19].

Based on the knowledge, this study was carried out to develop a multipex PCR by simultaneous detection of the four different genes, *slt* I, *slt* II, *uid* A and *eae* A, which are specific characteristics of *E coli* O157 : H7 and directly detect the bacteria from raw meats with the developed method.

Materials and Methods

Bacterial strains : Bacterial strains used in this study were shown in Table 1. Fifteen reference strains of *E coli* O 157 : H7 were obtained from *E coli* reference center of Pennsylvania State University, Pennsylvania, USA. The other strains of *E coli* and *Enterobacteriaceae* were obtained from the International *Escherichia* and *Klebsiella* Centre of the Statens Serum Institute, Copenhagen, Denmark. Also, four isolates of *E coli* from 40 raw beef samples in our laboratory were used.

Isolation and Identification of *E coli* from raw beef : Forty raw beef samples were collected from several meatplant during August 1996 in Korea and submitted for isolation and identification of *E coli* O157 : H7. Twenty-five gram of each sample was aseptically removed from each meat and minced after addition of 225ml modified EC broth (Merck, Darmstadt, Germany) containing 20μg/ml of novobiocin in a Stomach bag[9]. After the minced samples were incubated at 37℃ for 24h, the samples were subcultured on modified TSB(Merck, Darmstadt, Germany) for 24h, and then cultured on sorbitol MacConkey agar(SMAC; Merck, Darmstadt, Germany) and CT(cefixime and potassium tellulite)-

Table 1. Bacterial strains used in this study

Bacteria	No. of tested	Origin
E coli reference strains		
0157 : H7	15	USA*
0157 : H19	1	Denmark**
015 : H11	1	〃
020 : H-	1	〃
055 : H-	1	〃
078 : H28	1	〃
0111 : H-	1	〃
0114 : H32	1	〃
0119 : H27	1	〃
0148 : H28	1	〃
Klebsiella pneumoniae	1	〃
Salmonella cholerasuis	1	〃
dublin	1	〃
Citrobacter freundii	1	〃
Clostridium perfringens	1	〃
Yersinia enterocolitica	1	〃
Edwardsiella tarda	1	〃
Total	31	

* *E coli* 0157 : H7 were obtained from *E coli* reference center of Pennsylvania State University, Pennsylvania, USA.

**The other strains of *E coli* and *Enterobacteriaceae* were obtained from the International *Escherichia* and *Klebsiella* Centre of the Statens Serum Institute, Copenhagen, Denmark.

SMAC(Merck, Darmstadt, Germany). After incubation at 37℃ for overnight, about 20 sorbitol-negative colonies were selected from each plate and tested for lactose fermentation. Isolates that could ferment lactose within 24 hours were screened with antiserum against *E coli* O157. Biochemical test and serotyping with *E coli* O and H antisera(Denka Co. Japan) were further performed with isolates which were sorbitol negative and agglutinated with antiserum against *E coli* O157[1].

DNA extraction : The preparation of genomic DNA from the bacterium was performed by using CTAB(cetyl trimetyl ammonium bromide) method[20]. Briefly, the bacterial strains were cultured in LB broth overnight. The bacteria were harvested by centrifugation at 12,000 X g for 3 min. The harvested bacteria were resuspended in 567μl of TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0), and the solution was incubated at 37℃ for 1h after addition of 30μl of 20% sodium dodecyl sulfate and 3μl of proteinase K(20mg/ml in distilled water). After incubation of the reaction mixture with 100μl of 5M NaCl and 80μl of CTAB-NaCl solution (10% CTAB in 0.7M NaCl) at 65℃ for 10 min, the solution was extracted with phenol-chroform-isoamyl alcohol(25 : 24 : 1) solution and DNA was precipitated with isopropanol by incubation at -20℃ for 2h. The DNA was harvested by centrifugation at 12,000 X g for 20 min, washed with 70% ethanol, and resuspended in 30μl of TE buffer after vacuum dried. RNA was removed by incubation of the solution with RNase(1μg/ml) at 37℃ for 2h. The concentration of the DNA was measured at 260nm with a spectrophotometer (Pharmacia Biotech., Piscataway, N.Y, USA). The solution was kept at -20℃ until use.

The genomic DNA from raw beef samples was purified by boiling method[21]. Briefly, The minced meats, enriched meats in mEC at 37℃ for 24h and subcultured samples in mTSB after enrichment in mEC were clarified by centrifugation at 2,000 X g for 3 min.

The supernatant were harvested by centrifugation at 12, 000 X g for 10 min. The harvested bacteria were washed 3 times by centrifugation, and resuspended in 100μl of distilled water. The mixture was boiled for 10 min, and the pellets were removed by centrifugation at 12,000 X g for 10 min. The supernatant was used as template DNA in the PCR.

Polymerase chain reaction(PCR) : The primers for amplification of *slt* I, *slt* II, *uid* A(beta-glucuronidase) and *eae* A gene of *E coli* were designed based on informations obtained from previous reports[12,18] and synthesized with a DNA synthesizer(Applied Biosystems 392). The 100μl of PCR reaction mixture contained 2.5mM $MgCl_2$, 10mM Tris HCl(pH 8.3), 50mM KCl, 0.2mM of dNTPs(Gibco/BRL, Grand Island, NY, USA), 50pmole of each primer, 2.5 Units of *Taq* DNA polymerase(Gibco/BRL, Grand Island, NY) and 1μl to 5μl of DNA template in TE buffer. The reaction mixture was heated to 95℃ for 10 min followed by adding the *Taq* DNA polymerase, and by overlaying with 50μl of mineral oil. Amplification was performed in a GeneAmp PCR system (Model 9600, Perkin Elmer Co.) for 35 cycles. An amplification cycle was performed with denaturation at 94℃ for 1 min, annealing of primers at 60℃ for 1 min, and

extension at 72℃ for 1 min. Final extension was carried out at 72℃ for 10 min. Amplified PCR products were analysed by 1.5% agarose gel electrophoresis and visualized under UV illumination after staining with ethidium bromide.

Vero Cell Cytotoxic Assay : All *E coli* strains were tested for production of SLT I and SLT II by using Vero cell culture cytotoxic determination[9]. Briefly, culture filtrates obtained from the trypticase soy broth after incubation at 37℃ for 24h were tested for production of verotoxins. The filtrates were two-fold diluted with phosphate buffered saline. Vero cells obtained from the American Type Culture Collection were cultured in Eagle's minimum essential medium (EMEM ; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum(FBS) and gentamicin(25μg/ml). Two hundred μl of Vero cells(2.5×10^5cells/ml) in tissue culture medium with gentamicin and amphotericin B were placed in each well of a 96 well tissue culture plate(Costar, Cambridge, MA, USA) and grown in EMEM with 10% FBS at 37℃ for 24h. A 50μl aliquot of the culture filtrates was added in a well. After incubation at 37℃ in 10% CO_2 for 3 days, cytopathic effect(CPE) of Vero cells were examined. The titer of toxins produced from the bacteria was determined by final dilution showing 50% of CPE in the monolayers of the Vero cells.

Results

In order to develop a rapid and accurate method in dectetion of *E coli* O157 : H7 from raw beef, primers on 4 specific genes of the bacteria, *uid* A, *eae* A and *slt* I, II genes were designed based on informations from previous reports[12, 18] and synthesized with a DNA synthesizer. The sequences of the primers synthesized and used in this study are shown in Table 2.

The specificity of the multiplex PCR for *E coli* O157 : H7 was evaluated with 31 bacterial strains ; 13 SLT-producing *E coli* O157 : H7 strains, 2 SLT-negative *E coli* O157 : H7 strains, 1 SLT-producing *E coli* O111 : H-strain, 8 SLT-negative *E coli* strains, and several strains of other enteric bacteria. Analysis of 31 Enterobacteriaceae with the assay showed that all serotypes of *E coli* O157 : H7 were correctly identified with the *uid* A and *eae* A and/or *slt* I, *slt* II genes. The PCR products of 1,087bp(*eae* A), 584bp(*slt* II), 348bp(*slt* I) and 252bp(*uid* A) were successfully and simultaneously amplified in a single reaction, respectively(Fig 1). However, none of other serotypes of the *E coli* except O111 : H- and enteric bacteria was detected to possess the *uid* A, *eae* A and *slt* I, II genes with the PCR.

To determine the sensitivity of the multiplex PCR, three raw beef samples were artificially contaminated with from 10^1 to 10^8cells/g levels with *E coli* O157 : H7 reference strain. The minced beef samples were enriched in mEC supplemented with novobiocin(20μg/ml) and subcultured to mTSB to establish the multiplex PCR assay for direct detection of the presence of *E coli* O157 in raw meats. Also, the sensitivity of the multiplex PCR assay was compared with the direct meat enrichment in mEC and subculture in mTSB, respectively. All samples subcultured to mTSB after mEC en-

Table 2. Nucleotide sequence of primers used in this study

Primers	Nucleotide sequence('5-'3)	Target	PCR products(bp)
LP30	CAGTTAATGTGGTGGCGAAGG	*slt* I	348
LP31	CACCAGACAATGTAACCGCTG		
LP43	ATCCTATTCCCGGGAGTTTACG	*slt*II	584
LP44	GCGTCATCGTATACACAGGAGC		
PT2	GCGAAAACTGTGGAATTGGG	*uid* A	252
PT3	TGATGCTCCATCACTTCCTG		
AE19	CAGGTCGTCGTGTCTGCTAAA	*eae* A	1,087
AE20	TCAGCGTGGTTGGATCAACCT		

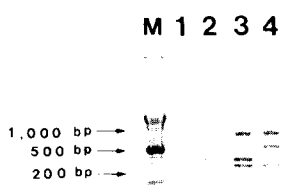

Fig 1. Specificity of the miltiplex-PCR for detection of *Escherichia coli* 0157 : H7.
The PCR products were analyzed by agarose gel(1.5%) electrophoresis followed by ethidium bromide staining. Lane M, DNA size marker(100bp ladder, BRL); 1, *E coli* 0157 : H7 strain(*slt*I$^+$ & *slt* II$^+$); 2, *E coli* 0157 : H7(*slt* I$^+$ & *slt* II^{++}); 3, *E coli* 0157 : H7(*slt*I$^+$); 4, *E coli* 0157 : H7(*slt* II^{++}, *E coli* 015 : H1; 6, *E coli* 020 : H-; 7, *E coli* 055 : H-; 8, *E coli* 078 : H28; 9, *E coli* 0114 : H32; 10, *E coli* 0119 : H27; 11, *E coli* 0148 : H28.

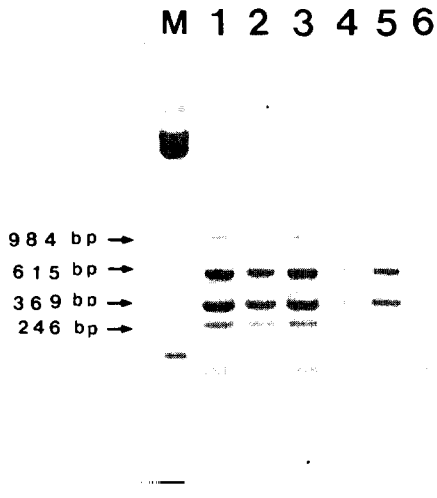

Fig 2. Sensitivity of the multiplex PCR assay for detection of *Escherichia coli* 0157 : H7 on subcultured meat samples in mTSB after enrichment on mEC. Lane M, DNA size marker (123bp ladder, BRL); 1- 5; *E coli* 0157 : H7(serial 10 - fold dilution; from 10^4 to 10^0 CFU/g), 6; negative control(No template DNA).

richment were detected as few as 10cells/g(Fig 2), otherwise more than 10^4cells/g could be detected before and after enrichment in mEC by the developed method. Therefore, the PCR method applied to meats cultured in mEC and mTSB could be more sensitive than those in mEC culture only. The detection limit of the *eae* A gene product was lower than *uid* A and *slt* gene products in the multiplex PCR assay.

Table 3. Comparison of the multiplex-PCR and verocytotoxic assay in *Escherichia coli* reference and field strains

E coli serotype	No. of strains	PCR assay				Cytotoxic for Vero cells
		slt I	*slt* II	*uid* A	*eae* A	
0157 : H7	9	+	+	+	+	+
0157 : H7	1	+	-	+	+	+
0157 : H7	3	-	+	+	+	+
0157 : H7	2	-	-	+	+	-
0157 : H19	1	-	-	-	-	-
015 : H11	1	-	-	-	-	-
020 : H-	1	-	-	-	-	-
055 : H-	1	-	-	-	-	-
078 : H28	1	-	-	-	-	-
0111 : H-	1	+	-	-	-	+
0114 : H32	1	-	-	-	-	-
0119 : H27	1	-	-	-	-	-
0148 : H28	1	-	-	-	-	-
O6	1	+	+	-	-	+
0112	1	+	+	-	-	+
0115	1	+	+	-	-	+
0139	1	+	+	-	-	+

As shown in Table 3, 13 reference strains of *E coli* O157 : H7 and 1 *E coli* O111 : H- were identified with the SLT types produced by each strain, and also confirmed by Vero cell cytotoxicity assay. The type of 4 SLT-producing *E coli* isolates from beef samples was identified by PCR assay apparently correlated with the Vero cell cytotoxicity assay.

In the application of the developed PCR method, four samples in 40 raw beef samples were positive for only *slt* I and *slt* II genes in the multiplex PCR assay(Fig 3). The isolates from 4 samples were identified as *E coli* O6, O112, O 115, O139 in serological test, respectively, but *E coli* O157 : H7 serotype was not detected from raw beef samples in Korea.

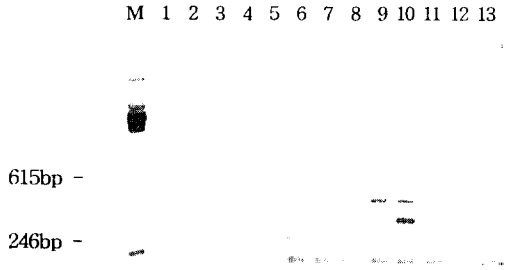

Fig 3. Application for detection of Shiga-like toxins producing *Escherichia coli* in beef samples by the multiplex-PCR method.
Lane M ; DNA size marker(100bp ladder, Promega), 1-13 : beef samples obtained from slaughter house.

Discussion

More than 250 food-borne disease are now recognized, and most of pathogens were required a specific laboratory diagnosis. *E coli* O157 : H7 has emerged as a major food-borne pathogen of considerable public health importance. Although *E coli* O157 : H7 is one of the most important enterohemorrhagic *E coli* (EHEC) and predominantly appears in most areas, many other *E coli* serotypes produce SLTs[2]. Usually, SLTEC or EHEC strains have been associated with food-borne diseases in human.

To detect SLTEC, immunological and DNA-based methods have been developed[8,16,22] However, at the beginning, most of the methods were targeted to SLTs or to the genes involved with virulence, but were not specific for *E coli* O157 : H7. Later on, oligonucleotide probe containing an unique base substitution in the allele of the *uid* A gene was applied to *E coli* O157 : H7 isolates[13,19]. Also, a multiplex PCR assay was developed to detect *E coli* O157 : H7 by simultaneous amplification of the *slt* I, *slt* II and *uid* A genes[18].

Enteropathogenic *E coli* (EPEC) strains also cause diarrhea in human as well as domestic animals, and produce an adhesin(intimin), but not SLTs. Intimin, a product of the *E coli eae*A gene, is also an important virulence factor both in EHEC and EPEC. Recently, genes associated with the development of AE lesions have been cloned and sequenced from EPEC and EHEC strains originated from human[23,24] The *eae* A genes of EHEC and EPEC share 97% homology at the 5' ends but are only 59% homologous over the last 800bp at the 3' terminal regions. One pair of primers with homology to the 3' nucleotide sequence of *eae* from *E coli* O157 : H7 appeared to be relatively specific for this O serogroup by PCR[12,25]. The primers could amplify DNA from all *E coli* O157 : H7 strains(H7 and H-) but not from other SLTEC or EPEC strains, with the exception of O serogroup 145 in SLTEC and O serogroup 55 in EPEC[25].

Based on the knowledge, an useful diagnostic test for EHEC and SLTEC should be able to detect *slt* I, II, *uid* A and *eae* A genes simultaneously. Therefore, in this study, we established the multiplex PCR assay as a rapid and specific test for detection of *E coli* O157 : H7.

Fifteen reference strains of *E coli* serotype O157 : H7 were simultaneously amplified in the multiplex PCR assay with the *uid* A and *eae* A and/or *slt* I, II primers. However, none of other serotypes of the *E coli* except O111 : H- and enteric bacteria were detected to possess the *uid* A, *eae* A and *slt* I, II genes by the PCR assay(Fig 1). The results have shown that the multiplex PCR could identify the type of SLT, and confirm the *eae* gene associated with virulence factor, and at the same time, differentiate specifically other SLTEC from *E coli* O157 : H7.

E coli O157 : H7 has relatively low infectious dose, therefore, illness can be occurred after ingestion of less than 100 organisms[2,3]. In this study, samples subcultured to mTSB aft-

er mEC enrichment were detected as few as 10 cells/g of *E coli* O157 : H7(Fig 2) by the multiplex PCR assay. The result of this study has indicated that the developed PCR method is significantly sensitive to detect the microrganisms present below the virulent level in meats.

Three Shiga-like toxin producing *E coli* O157(0.78%) have been isolated from 390 fecal samples of cattle in Korea[26]. However, there was no report on *E coli* O157 : H7 infection in human, and also not detected from meats in the country. Those reports suggested that the distribution of *E coli* O157 : H7 might be very rare in domestic animals as well as in human in Korea. Although *E coli* O157 : H7 is a common serotype of EHEC, a number of other *E coli* serotypes including O26 : H11, O111 : H8, O113 : H21, O145 : NM also cause HUS and hemorrhagic colitis in human[27]. Only four samples of 40 raw beefs were positive for *slt* I and *slt* II genes by the PCR method in this study, and the 4 isolates from beef samples were identified as *E coli* O6, O 112, O115, O139 in serological test, respectively. Numerous serogroups of *E coli* isolates have been recognized as producers of SLTs which can cause food poisoning in human[3]. Foods of animal origin, principally from cattle, have been linked as vehicles of SLTEC and EHEC. So, the proper hygienic practices should be applied to handling foods originated from animal to prevent the food-borne diseases caused by SLTEC and EHEC.

These results suggested that the developed PCR could be applied as a sensitive, specific and rapid method for the detection of *E coli* O157 : H7 by simultaneous amplification of virulence genes(*slt* I, II, and *eae* A) as well as a specific *uid* A gene of the bacteria. Furthermore, the PCR method could be used to identify and differentiate the EHEC, SLTEC and EPEC strains in epidemiology.

Conclusion

This study was carried out to establish a multiplex polymerase chain reaction(PCR) which could simultaneously amplify *slt* I, *slt* II, *uid* A and *eae* A genes of *Esherichia coli* O157 : H7 and detect the bacteria directly from raw meats with the method.

In the analysis of 31 *Enterobacteriaceae* with the multiplex PCR, the PCR products, 1,087bp(*eae* A), 584bp(*slt* II), 348bp(*slt* I) and 252bp(*uid* A), were specifically and simultaneously amplified by the PCR method and all reference strains of *E coli* serotype O157 : H7 were identified by amplification of the *uid* A, *slt* I, II or *eae* A genes from these strains.

Regardless modified EC enrichment, the bacteria were detected from the highly contaminated samples(more than 10^4 cells/g) by the developed mutiplex PCR. However, subculture on modified TSB following mEC enrichment was required to detect the bacterium in samples as few as 10 cells/g.

The cytotoxicity of the *E coli* O157 : H7 reference strains possessed either *slt* I or *slt* II, or both genes was confirmed by Vero cell cytotoxicity assay. Correlation between result of the PCR and Vero cell cytotoxicity was confirmed using 4 SLT-producing *E coli* isolated from 40 beef samples.

This study indicated that the developed multiplex PCR is a specific, sensitive and rapid method for the detection of *E coli* O157 : H7 directly from beef samples.

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