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Isolation and identification of *Escherichia coli* O157:H7 using different detection methods and molecular determination by multiplex PCR and RAPD

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***Escherichia coli* O157:H7 is recognized as a significant food-borne pathogen, so rapid identification is important for food hygiene management and prompt epidemiological investigations. The limited prevalence data on Shiga toxin-producing *E. coli* (STEC) and *E. coli* O157:H7 in foods and animals in Korea made an assessment of the risks difficult, and the options for management and control unclear. The prevalence of the organisms was examined by newly developed kit-*E. coli* O157:H7 Rapid kit. For the isolation of *E. coli* O157:H7, conventional culture, immunomagnetic separation, and *E. coli* O157:H7 Rapid kit were applied, and multiplex PCR and randomly amplified polymorphic DNA (RAPD) were performed for the molecular determination. There was high molecular relatedness among 11 Korean isolates and 17 U. S. strains at 63% level. Additionally, distinct differentiation between pig and cattle isolates was determined. It implied that RAPD had a capacity to distinguish strains with different sources, however it could not discriminate among isolates according to their differences in the degree of virulence. In antimicrobial susceptibility tests, 45.5% of isolates showed antibiotic resistance to two or more antibiotics. Unlike the isolates from other countries, domestic isolates of *E. coli* O157:H7 was mainly resistant to ampicillin and tetracylines. In summary, the application of *E. coli* O157:H7 Rapid kit may be useful to detect *E. coli* O157:H7 due to its sensitivity and convenience. Moreover, combinational analysis of multiplex PCR together with RAPD can aid to survey the characteristics of isolates.**

Key words: *Escherichia coli* O157:H7, multiplex PCR, RAPD

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Introduction

Shiga toxin-producing *Escherichia coli* (STEC) has been recognized as an important cause of human diseases such as hemolytic uremic syndrome (HUS) [29,36]. STEC constitute one of the most important causes of food-borne disease worldwide. Since the first report by Riley *et al.* [38], STEC has been associated with outbreaks and sporadic cases of human diseases, ranging from uncomplicated diarrhea to hemorrhagic colitis and HUS. Disease in humans following infection with STEC generally results in either exclusively intestinal symptom, such as abdominal pain, and bloody or nonbloody diarrhea, or less frequently, serious systemic complications. The complications associated with STEC infection are largely related to the development of thrombotic microangiopathy in a number of sites. This is especially prevalent in the kidney, and the end result is the development of HUS, which is characterized by the triad of acute renal failure, thrombopenia, and anemia. A number of organs other than the kidney are often involved in STEC-related complications. Central nervous system and pancreas are frequent targets [1]. Besides humans, STEC can cause damage to animals. For example, STEC develops renal tubular necrosis in mice and damages certain endothelial cells in pigs and rabbits. Greyhounds inoculated with STEC develop vascular lesions in the glomeruli that mimic those seen in patients with HUS [3].

STEC has been found to produce a family of related cytotoxins known as Shiga toxins (Stxs). They have been classified into two major classes, Stx1 and Stx2. Whereas the Stx1 family is very homogenous, several Stx2 variants have been identified. These variants are: Stx2c and Stx2d produced by human STEC isolates, Stx2e typically found in STEC pathogenic for pigs, and Stx2f, described recently in STEC isolates from feral pigeons [40]. An STEC can produce Stx1, Stx2 (or its variants) or both. The Stx2 is

responsible for the severe necrotic renal tubular lesions and death of treated mice fed an EHEC which possesses both Stx1 and Stx2. This difference in toxicity is also evident when human renal microvascular endothelial cells are treated with purified Stx1 or Stx2. They are capable of crossing an intact polarized epithelium via an energy-requiring process and, most importantly, the toxin that moves across this barrier retains its biological activity; damage to epithelial cells. Except Stxs, there are several virulence factors can contribute to the pathogenicity in STEC. The *eae* gene that codes intimin is a 94-to 97-kDa outer membrane protein produced by all attaching-and-effacing (A/E) enteric pathogens including STEC O157:H7. It is the only bacterial adherence factor identified thus far as important intestinal colonization in animal models. Another putative virulence factor is RTX toxin designated as EHEC-hemolysin, coded by the EHEC *hly* operon. There are two different plasmid-encoded hemolysins, both members of the RTX toxin family, have been described for STEC. Alpha-hemolysin is formed by porcine edema disease-causing STEC serovars which produce Stx variant 2e. Moreover, STEC serotypes may also possess additional virulence factors such as secreted proteins for signal transduction encoded by *esp*A, *esp*B and *esp*D and the translocated intimin receptor encoded by *tir* [7].

STEC infection has been often associated with the consumption of contaminated ground beef, raw milk, and other bovine products, thus cattle are suspected to be a primary reservoir [15]. But bacteria also have been isolated from domestic [6] and wild animals [48]. Moreover, recent outbreaks of foodborne illness associated with eating fresh products have heightened concerns that these foods contaminated with STEC may be an increasing source of illness [43]. In the past decades, outbreaks of diseases caused by STEC have been associated with the consumption of leaf lettuce [2], potatoes [9], radish sprouts [50], and raw vegetables [34]. Fruit-related outbreaks have also been caused by the consumption of fresh-pressed apple juice [13].

Detection of *E. coli* O157:H7 in the clinical laboratory is dependent on distinguishing the pathogenic serotypes from normal fecal flora containing commensal strains of *E. coli*. Fortunately, *E. coli* O157:H7 has two unusual biochemical markers; delayed fermentation of *D*-sorbitol and lack of β-D-glucuronidase activity, which help to phenotypically separate O157:H7 isolates from nonpathogenic *E. coli* strains [49]. One of these markers (delayed sorbitol fermentation) enables to develop several selective media (e.g., Sorbitol-MacConkey; SMAC) which aid in the initial recognition of suspicious colonies isolated from bloody stools. The selectivity of SMAC agar has been improved with the addition of cefixime-rhamnose (CR-SMAC), cefixime-tellurite (CT-SMAC), and 4-methylumbelliferyl-β-D-glucuronide (MSA-MUG). In addition to modifying of SMAC agar, new selective media have been developed to increase the effectiveness of *E. coli* O157:H7 isolation, including Fluorocult *E. coli* O157:H7 (Merck, Germany), Chromocult agar (Merck, Germany), Rainbow agar O157 (RB; Biolog, USA), and Biosynth Culture Media O157:H7 (BCM O157:H7; Biosynth, Switzerland). Once suspicious colonies are identified, confirmation of the isolates as *E. coli* O157:H7 is dependent upon biochemical identification and demonstration of the presence of somatic and flagellar antigens (O157, H7). These steps are necessary since other enteric bacteria can be sorbitol-negative and can possess antigens those are identical to or cross-reactive with O157 antigens. However, Feng [16] reported that sorbitol-fermenting *E. coli* O157:H7 had been detected from foods and increased number of such strains has been identified in Europe. Furthermore, an increasing phenotypic variation in O157 isolates has been noted in European studies which could potentially lead to misidentification of O157:H7 as some other species [49].

Detection of *E. coli* O157:H7 from food samples requires enrichment and isolation with selective and/or indicator media, but lacks specificity to identify STEC [36,39,53]. Thus, more sensitive methods are required to improve the detectability of STEC O157:H7 from food and environmental samples. Apart from the traditional culture methods relying on biochemical characteristics, various genotypic methods have been proven useful for species identification, epidemiological typing, and determining genetic relatedness among pathogenic and nonpathogenic bacteria [44]. Besides, the low infectious dose of *E. coli* O157:H7 (from 50 to 100 organisms) necessities the development of sensitive detection techniques. For examples, immunomagnetic separation (IMS) techniques have been employed widely within routine microbiology testing laboratories for the isolation of specific microorganisms [9,20]. IMS allows the rapid capture and concentration of bacteria from a range matrics. The magnetic beads used for IMS are commercially available, either pre-coated with antibodies or ready for antibody conjugation. The beads are typically 2-3 μm spheres containing Fe_2O_4 and Fe_3O_4 to make them super-paramagnetic. They are only magnetic in the presence of a magnetic field and readily separate from each other when the magnetic field is removed. By applying a strong magnetic field to the outside of the reaction vessel, the beads and captured bacteria can be immobilized against the vessel wall. This allows selective removal of the remainder of the samples including non-target bacteria and other organic particles. The beads are then released by withdrawing the magnet. This simple step of IMS procedure can help us to isolate STEC from samples easily. Recently, immunomagnetic particles for the separation of *E. coli* O26 and O111 have become commercially available. With the use of IMS, the isolation rate of *E. coli* O157 has been markedly improved. Wright *et al.* [51] showed a 100-fold increase in sensitivity of detection by IMS compared with direct subculture from

enrichment broth. However, manual IMS (MIMS) is very labor intensive when large numbers of samples have to be analyzed. So, an automated IMS in combination with an integrated ELISA (EiaFoss; Foss, Denmark) would increase efficiency and lighten the workload. This method can test about 81-108 samples per day, after overnight enrichment [37]. The latex agglutination method (Verotox F-Assay) for the Stxs detection has been developed and available [24]. It is based on the use of latex particles sensitized with antibodies to these two toxins which are detected by reversed passive latex agglutination (RPLA). Additionally, methods to detect Stx-gene or Stx-production have been proven to be useful for identification of STEC. Among a lot of commercially available detection techniques, we selected one of visual immunochromatographic assays, *E. coli* O157:H7 Rapid kit (Dong-A Pharm, Korea). The effectiveness of the kit has not yet been determined. We examined its capacity to detect STEC O157:H7 comparing with IMS which is proven to be one of the most sensitive detection techniques.

The isolation of *E. coli* belonging to serogroup O157 has rarely been reported in Asian countries except Japan; though isolation of *E. coli* O157 from clinical sources in India, China, Korea, and Hong Kong has been briefly reported [47]. The limited prevalence data on STEC and *E. coli* O157:H7 in foods and animals in the country made an assessment of the risks difficult, and the options for management and control unclear.

The objectives of this study are (i) to examine the prevalence of *E. coli* O157:H7 in slaughterhouses and retail markets, (ii) to characterize the isolates by determination of *stx*1, *stx*2, *eae*A, and *hly*A in multiplex PCR assay, (iii) to compare the genetic patterns of Korean isolates and U.S. isolates, and (iv) to compare the efficiency among conventional culture method, IMS, and *E. coli* O157:H7 commercial diagnostic kit, the *E. coli* 0157:H7 Rapid kit.

The study will provide information on newly developed diagnostic kit for its detectability, rapidity and convenience to perform. The diagnostic procedures examined in this study can be correctly applied to the areas which require to supervise the presence of the organism, especially enforced the Hazard Analysis Critical Control Point (HACCP) program. And, the result of genotypes of the isolates can envision the determination of Korean epidemiological characteristics. All together, we may propose the effective control strategy against STEC infection in humans and animals, and food contamination in livestock products.

Materials and Methods

Bacterial strains

E. coli O157:H7 strains used in this study are listed in Table 1. Four strains, one produces both Stx1 and Stx2, and one produces Stx1 only, one produces Stx2 only, and one non-Stx producing strain, were obtained from American Type Culture Collection (ATCC). Seven *E. coli* O157:H7 strains were obtained from *E. coli* reference center (Pennsylvania State University, USA) and six strains were obtained from Cornell University. Additionally, eleven Korean isolates detected in this study were also listed.

Sample collections

From April 2000 to June 2002, a total of 1,682 samples were collected. Among them, 1,042 fecal samples were collected from pigs and cattle at 3 slaughterhouses, and from chicken at meat processing plants. The sponge sampling method was used to collect 286 pork and beef samples and homogenization was conducted to process the samples from retail markets. A total of 355 chicken samples were obtained from chicken meat processing plants and markets by rinsing the samples with buffered peptone water (BPW; Becton Dickinson, USA).

In case of fecal samples, a cup of feces was taken into each 100 ml of specimen cup, and pork and beef carcasses from three slaughterhouses were conducted by sponge sampling method within 24 h after slaughtering [19]. For each carcass, three sites were investigated; belly, leg, and hip. For swabbing with sterilized sponge, an area of 5 by 10 cm was delimitated by sterile plastic template. The delimited area was then swabbed with a sterilized sponge that had been moistened by being placed in a sterilized vial with 10 ml of BPW in Meat/Turkey Carcass Sampling Kit

Table 1. Bacterial strains used in this study

Sample No.[a]	Bacterial strains	Stxs genes[b]	Sources
A1	43888	- , -	ATCC
A2	43889	- , +	ATCC
A3	43892	+ , -	ATCC
A4	43894	+ , +	ATCC
C1	29 (4-FS)	+ , -	Cornell U.
C2	40 (1398)	- , -	Cornell U.
C3	41 (973)	- , -	Cornell U.
C4	42 (75)	+ ,+	Cornell U.
C5	43 (796)	+ ,+	Cornell U.
C6	44 (1489)	- , +	Cornell U.
P1	3009-88 (3D)	+ , +	Penn. Univ.
P2	3077-88 (3E)	- , +	Penn. Univ.
P3	3104-88 (3C)	+ , +	Penn. Univ.
P4	3299-85 (3A)	+ , +	Penn. Univ.
P5	C7-88 (4E)	+ , -	Penn. Univ.
P6	C681-87 (4D)	- , +	Penn. Univ.
P7	C999-87 (4B)	- , -	Penn. Univ.

[a]Strains: A1-4 (ATCC strains), C1-6 (strains of Cornell Univ.) and P1-7 (strains from *E. coli* reference center of Pennsylvania State Univ.)
[b]The presence of Stx1 and Stx2. "-" and "+" indicate negative and positive, respectively.

(Nasco, USA), and placed into the icebox. Upon arrival at the laboratory, samples were either analyzed immediately or held at 4°C for no longer than 24 h before analysis. Each sample was placed aseptically in a stomacher bag with 90 ml BPW and mixed using a stomacher and incubated at 37°C for 6 h and 24 h. In case of meat samples from retail markets weighed 25 g, then aseptically transferred into sterile plastic bags (Whirl-Pak, Nasco, USA) and were held at 4°C. After arrival, samples were homogenized with 225 ml of BPW, and incubated at 37°C for 6 h and 24 h.

Chicken samples were obtained from two chicken meat processing plants. Chicken carcasses were collected from the line at a processing plant after rinsing inside and outside and immediately before entering the chill tank. All carcasses had been eviscerated, inspected, and subjected to repeat wash steps. Each carcass was placed into an individual sterile plastic bag with 400 ml of BPW. To obtain carcass rinse, each carcass was massaged thoroughly for 3-5 min. Then, only 50 ml of the broth was taken in the conical tube (Becton Dickinson, USA), and placed into the ice for transport to the laboratory within 4 h. Ten ml of each sample was transferred into 90 ml of BPW for preliminary enrichment.

Enrichment Procedures

As described above, 6 h-incubation broth with BPW was used directly for analysis of IMS. On the other hand, 24 h-incubation broth with BPW was used for conventional culture method and analysis of the *E. coli* O157:H7 Rapid kit. After 24 h-incubation, 10 ml of each broth was transferred into 90 ml of modified *E. coli* broth (mEC; Becton Dickinson, USA) supplemented with novobiocin (20 mg/l) (Difco, USA) for secondary selective enrichment.

Analysis of *E. coli* O157:H7 using IMS

One milliliter portions of the enriched homogenate were mixed with 20 µl magnetic polystyrene beads coated with *E. coli* O157 antibody (Dynabeads, Norway). Separation and washing procedures were followed by the manufacturers instructions. Washed beads were resuspended in 100 µl wash buffer and 50 µl were streaked on SMAC agar supplemented with cefixime (0.05 mg/l) and tellurite (2.5 mg/l, CT-SMAC, Dynabeads, Norway). CT-SMAC plates were incubated at 37°C for 18-24 h and sorbitol-negative colonies were streaked for confirmation on Chromocult agar (Merck, Germany), which were held at 37°C overnight. These presumptive *E. coli* O157 isolates were tested for motility test and agglutination test with O157 and flagellar H7 antiserum (Difco, USA). For motility test, overnight cultured colonies were inoculated into motility test medium (Difco, USA) and incubated at 37°C for 24 h. This experiment was repeated 3 times for increase motility of isolates. And, their biochemical properties were determined using API 20E (BioMérieux, France). Agglutinating strains which were serotyped (O157 and H7 antigen) were performed multiplex PCR for identifying the presence of several virulence factors.

Conventional Culture Method

After secondary selective enrichment procedures with 90 ml of mEC broth, one loopful of the broth was inoculated onto CT-SMAC agar. After 24 h- incubation at 37°C, up to five colorless colonies were transferred onto Chromocult agar and incubated at 37°C overnight. The purple colonies were examined by the standard biochemical tests for confirmation of *E. coli* [22]. Those identified as *E. coli* were subjected to motility test and the slide agglutination test using anti-O157 and flagellar H7 serum as described in IMS. Presence of virulence genes was examined by the multiplex PCR method.

Analysis with the *E. coli* O157:H7 Rapid kit

For the *E. coli* O157:H7 Rapid kit assay, 100 µl of secondary enrichment broth culture (as mentioned above) was added to the sample well and incubated at room temperature for 5-10 min before recording results. Results of the assays were interpreted according to the manufacturer's instructions. The *E. coli* O157:H7 positive strains were applied for further determination by multiplex PCR and PCR for flagellar H7 antigen detection.

DNA preparation for Multiplex PCR, flagellar H7 PCR and RAPD analysis

E. coli O157:H7 strains which isolated from three experiments used in this study were cultured on 5% sheep blood agar (Korea Media, Korea). The USA standard strains and ATCC strains were also cultured on 5% sheep blood agar. After overnight culture, suspected colonies from each plate were inoculated into Tryptic Soy Broth (TSB; Difco, USA), and the broth was incubated at 37°C for 24 h. Boiling method was used to obtain DNA template as previously described [36]. One-milliliter aliquot of broth culture was centrifuged at 12,000 rpm for 5 min, and the supernatant was discarded. The cell pellet was resuspended in 1.0 ml of sterile distilled water. Cells were boiled for 15-20 min, and the insoluble material was removed by centrifugation for 5 min. The supernatant was collected and used as a template.

Multiplex PCR for *stx*1, *stx*2, *eae*A, and *hly*A, and the flagellar H7 gene amplification

Multiplex PCR for the detection of *stx*1, *stx*2, *eae*A, and EHEC *hly*A gene was performed by a GeneAmp PCR thermocycler (Model 2400, Perkin-Elmer, USA). Oligonucleotide primers for *Stx*1, *Stx*2, *eae*A, and *hly*A were synthesized as previously described [14]. Oligonucleotide sequence of primers and the predicted sizes of PCR amplified products are listed in Table 2. Each primer pair

had been determined to be specific for *E. coli* and had been shown not to amplify products detectable by agarose gel (Sigma, USA) electrophoresis using DNA templates derived from a range of Gram-positive and Gram-negative bacterial species from various food and animal sources.

PCR assays were carried out in a 50 µl volume containing 4 µl of nucleic acid templates prepared from cultures and reference strains. And 10 mM Tris-HCl (pH 8.4), 10 mM KCl, 3 mM $MgCl_2$; 20 pmol concentrations of each primer, 0.2 mM dNTPs, and 1 U of *Taq* DNA polymerase (Promega, USA) were added to the reaction mixtures. PCR conditions consisted of an initial 95°C denaturation step for 3 min followed by 35 cycles of 95°C for 20 s, 58°C for 40 s, and 72°C for 90 s. The final extension cycle was followed by at 72°C for 5 min. Amplified DNA fragments were resolved by gel electrophoresis using 1.5% agarose gels in Tris-acetate-EDTA (TAE) buffer. Gels were stained with 0.5 µl of ethidium bromide (EtBr) per ml, visualized and photographed under UV illumination.

Another PCR amplification analysis was executed for confirmation of the presence of the flagellar H7 gene. The PCR primers for H7 were previously described by Gannon *et al.* [18]. Oligonucleotide sequence of the primer and expected sizes were listed in Table 2. The flagellar H7 PCR assay was performed in 100 µl reaction volume containing 2.5 U *Taq* DNA polymerase (Promega, USA), 0.2 mM of dNTPs, 2.5 mM $MgCl_2$, 50 mM KCl, and 20 pmol of flagellar H7 primer. The reactions were carried out with a GeneAmp PCR thermocycler. The PCR condition was at 94°C for 1 min, 65°C for 2 min, and 72°C for 2 min. The final extension cycle was followed by at 72°C for 5 min. The amplified PCR products were separated on 1.5% agarose gels in TAE buffer, followed by EtBr staining and photographed under UV illumination.

RAPD fingerprinting

To increase the reproducibility of RAPD analysis, two kinds of 10-mer random primers (referred as CRA22 and CRA23) were used for investigation of *E. coli* O157:H7 isolates and reference strains. Based on the results obtained, primer CRA22 and CRA23 were commercially synthesized for analysis of *E. coli* O157:H7 strains. Twenty ng of each primer with 70% G+C content resulted in complicated and unrepeatable PCR band patterns [31]. Two primers, CRA22 and CRA23, were combined in equimolar ratios and used at 20 pmol per primer per 100 µl reaction mixture. Amplification reactions were performed in a total volume of 100 µl containing 3 mM $MgCl_2$, 0.2 mM each dNTPs, 20 pmol of each PCR primer, 2 U of *Taq* DNA polymerase (Takara, Japan), and 10 µl of templates. Temperature conditions consisted of an initial 94°C denaturation step for 4 min followed by 30 cycles of 94°C for 20 s, 45°C for 30 s, and 72°C for 1 min. The final extension cycle was followed by at 72°C for 10 min. The reaction was conducted with GeneAmp PCR thermocycler. PCR products were resolved 1% agarose gel in TAE buffer. Agarose gel was stained in EtBr solution (0.5 mg/ml) to visualize amplified DNA bands. The banding patterns generated by RAPD-PCR and genetic distances between strains were analyzed with a Quantity-One program with Gel-Doc (Bio-Rad, USA). In addition, the discriminatory power was determined according to the numerical index method described by Hunter and Gaston [23]. The *D*-value indicates that two isolates randomly selected from the test population will be assigned to different typing groups. The formula of *D*-value is as follows.

$$D = 1 - 1/N(N-1)\sum_{j=1}^{S} n_j(n_j - 1)$$

S = total number of different types, n_j = number of isolates representing each type and N = number of isolates within the test population. Overall flow-chart from sampling to RAPD was shown in Fig 1.

Table 2. Primers used in multiplex PCR, flagellar H7 PCR, and RAPD fingerprinting assay

Primer	Oligonucleotide sequences (5'-3')	Expected size	Reference
*stx*1-F	ACACTGGATGATCTCAGTGG	614 bp	Fagan *et al* [14]
*stx*1-R	CTGAATCCCCCTCCATTATG		
*stx*2-F	CCATGACAACGGACAGCAGTT	779 bp	Fagan *et al* [14]
*stx*2-R	CCTGTCAACTGAGCAGCACTTTG		
*eae*A-F	GTGGCGAATACTGGCGAGACT	890 bp	Fagan *et al* [14]
*eae*A-R	CCCCATTCTTTTTCACCGTCG		
*hly*A-F	ACGATGTGGTTTATTCTGGA	165 bp	Fagan *et al* [14]
*hly*A-R	CTTCACGTGACCATACATAT		
H7-F	GCGCTGTCGAGTTCTATCGAGC	625 bp	Gannon *et al* [18]
H7-R	CAACGGTGACTTTATCGCCATTCC		
CRA22	CCGCAGCCAA		Neilan *et al* [31]
CRA23	GCGATCCCCA		Neilan *et al* [31]

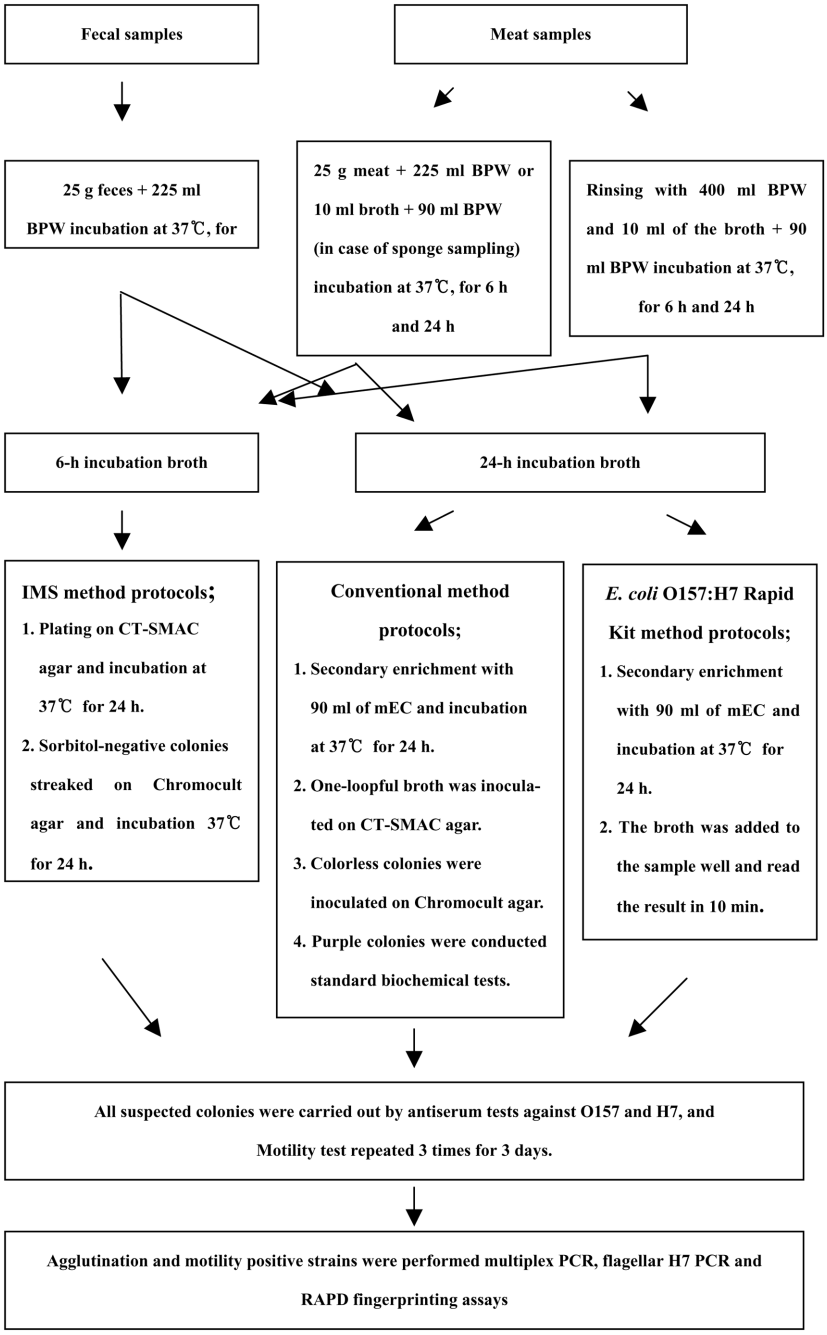

Fig. 1. Procedures for the isolation of STEC from fecal and meat samples.

Vero cell cytotoxic assay

After confirmation of *E. coli* O157:H7 from isolates in this study by multiplex PCR and flagellar H7 PCR, the isolates were carried out by Vero cell cytotoxic assay to characterize them. The assay was conducted as previously described by Yoh *et al.* [52] and Kim *et al.* [26]. Briefly, culture filtrates obtained from the TSB after incubation at 37°C for 24 h were used for the assay. Culture supernatants and extracts were filtered through 0.2 μm pore-size sterile filter (Minisart; Sartorius, Germany). Vero cells were cultured in Eagles minimum essential medium (EMEM; Gibco, USA) supplemented with 10% fetal bovine serum (FBS) and gentamicin (100 μg/ml). Two-hundred μl of Vero cells in EMEM (2.5×10^5 cells/ml) were placed in each well of 96 well tissue culture plate (Costar, USA) and incubated at 37°C for 24 h. Fifty μl of aliquot of the culture filtrates was added into each well. After incubation at 37°C in 10% CO_2 atmosphere for 3 days, the cytopathic effect (CPE) on

Vero cells was examined under an inverted microscope (DMIRB/E; Leica, Germany). In this study, we determined that "weak" was ranging from 0% to 30%, and "strong" was from 30% to 100% of Vero cells were dead. The result was shown in Table 5.

Antimicrobial susceptibility test

The antimicrobial susceptibility of 11 *E. coli* O157:H7 isolates was determined by Bauer and Kirby method [5]. A total of 23 concentrated antimicrobial discs tested were ampicillin (10 μg), amikacin (30 μg), amoxicillin/clavulanic acid (20/10 μg), carbenicillin (100 μg), cefixime (5 μg), cefotaxime (30 μg) cephalothin (30 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), erythromycin (15 μg), gentamicin (10 μg), imipenem (10 μg), kanamycin (30 μg), levofloxacin (5 μg), nalidixic acid (30 μg), norfloxacin (10 μg), ofloxacin (5 μg), polymyxin B (300 U), sparfloxacin (5 μg), streptomycin (10 μg), tetracycline (30 μg), tobramycin (10 μg), and trimethoprim/sulfamethoxazole (1.25/23.75 μg). All antimicrobial discs are purchased from Becton Dickinson (USA). After 24 h-incubation in TSB, isolates subcultured in Muller-Hinton broth (MHB, Difco, USA) for 8 h, diluted to MacFarland scale No. 0.5, and applied to the surface of Muller-Hinton Agar (MHA, Difco, USA). The discs were placed using disc dispenser (Becton Dickinson, USA) and the plates were incubated for 18 h at 37°C. Inhibitory zones of the growth were measured. The results were interpreted by the guideline of National Committee for Clinical Laboratory Standards (NCCLS).

Results

Isolation of *E. coli* O157:H7

In this study, a total of 1,682 samples were examined. Nine *E. coli* O157:H7 were isolated from fecal samples, and two were obtained from meat samples. However, no *E. coli* O157:H7 was detected from chicken rinsing samples.

The detection rates of *E. coli* O157:H7 by the three different methods were different (Table 3). In conventional method, seven isolates were obtained through phenotypical characteristics (non-sorbitol fermenters forming colorless colonies on CT-SMAC agar and purple colonies on Chromocult agar). The 11 isolates were detected by the *E. coli* O157:H7 Rapid kit and 10 suspected isolates in IMS were further applied to motility and agglutination tests. In agglutination and motility tests, strains isolated from same samples showed identical results regardless of different isolation methods. At motility test, all eleven strains were positive. In agglutination test against O157 antiserum, all strains showed positive, but one of them did not agglutinate against H7 antiserum.

Characterization of *E. coli* O157:H7 isolates by multiplex PCR for *Stx*1, *Stx*2, *eae*A, and *hly*A genes, and by flagellar H7 PCR

After identification by motility and agglutination tests

Table 3. The detection rates of *E. coli* O157:H7 by three different methods

Methods	Positive (%)	Negative (%)
Conventional culture	0.42 (7/1,682)[a]	99.58 (1,675/1,682)[b]
Immunomagnetic separation	0.59 (10/1,682)	99.41 (1,672/1,682)
E. coli O157:H7 Rapid kit	0.65 (11/1,682)	99.35 (1,671/1,682)

[a]No. of positive/No. of samples examined
[b]No. of negative/No. of samples examined

Table 4. Antibiotic resistance profiles of isolated *E. coli* O157:H7

Antimicrobial discs	Resistant (%)	Intermediate (%)	Antimicrobial discs	Resistant (%)	Intermediate (%)
Ampicillin	27.2	54.5	Kanamycin	0	27.3
Amikacin	0	0	Levofloxacin	0	0
Amoxicillin/ clavulanic acid	9.1	45.5	Nalidixic acid	0	9.1
			Norfloxacin	0	0
Carbenicillin	9.1	72.7	Ofloxacin	0	0
Cefixime	0	0	Polymyxin B	0	36.4
Cefotaxime	0	18.2	Sparfloxacin	0	0
Cephalothin	18.2	27.3	Streptomycin	0	36.4
Chloramphenicol	0	0	Tetracycline	18.2	36.4
Ciprofloxacin	0	0	Tobramycin	0	0
Erythromycin	100	0	Trimethoprim/ sulfamethoxazole	0	0
Gentamicin	0	0			
Imipenem	0	0			

Table 5. Results of multiplex PCR, H7 PCR, antiserum tests, motility test, and vero cell assay

Isolates	Presence of[a]				Antiserum tests[b]			Motility Test[b]	Verocell Assay[c]
	*stx*1	*stx*2	*eae*A	*hly*A	H7	O157	H7		
P010726-18	+	-	+	+	+	+	+	+	++
P010726-21	+	+	+	+	+	+	-	+	+
P010726-22	+	+	+	+	+	+	-	+	+
P010726-23	+	-	+	+	+	+	+	+	++
P010726-24	+	-	+	+	+	+	+	+	++
P010726-25	+	+	+	+	+	+	+	+	++
P010726-26	+	-	+	+	+	+	+	+	++
E010206-13-2	+	-	+	+	+	+	+	+	++
J010303-11-1	+	+	+	+	+	+	+	+	++
O157-R1-3-2	+	+	+	+	+	+	-	+	++
O157-C-1-2	+	-	+	+	+	+	+	+	+

[a] +; present, -; absent.
[b] +; positive, -; negative.
[c] ++; strong cytopathic effect (CPE), +; weak CPE.

against O157 and H7 antiserum, multiplex PCR and flagellar H7 PCR were carried out using primers for *stx*1, *stx*2, *eae*A, and *hly*A genes. As shown in Table 5, all eleven had *stx*1 genes, while six isolates had *stx*2 genes. Eleven isolates were confirmed as *E. coli* O157:H7 because they all carried *eae*A and *hly*A genes. Specific amplicon sizes of *stx*1, *stx*2, *eae*A, and *hly*A genes were 614 bp, 779 bp, 890 bp, and 165 bp, respectively. The PCR products representing each of four target STEC virulence factors were amplified with standard strain, ATCC 43894 as a positive control (lane 12 in Fig. 2).

After confirmation by motility and antiserum tests, the isolates were further applied to flagellar H7 assay and multiplex PCR assay to confirm the presence of flagellar gene. In flagellar H7 PCR assay, all eleven isolates were found harboring H7 genes. Though one isolate did not react against H7 antiserum, they all possessed H7 genes (Table 5).

RAPD fingerprinting analysis

Eleven *E. coli* O157:H7 isolates were compared with the 17 *E. coli* O157:H7 strains which were obtained from ATCC (4 strains), Cornell University (6 strains), Pennsylvania State University (7 strains) using RAPD assay. Representative RAPD patterns for all 28 tested strains amplified with two primers each (CRA22 and CRA23) were shown in Fig. 3. DNA polymorphism in the isolates was most evident amongst amplicons in the 2501 bp, 500 bp region. Fig. 3 illustrated a dendrogram constructed from amplicon profiles generated by CRA22 and CRA23. The dendrogram also contained 5 groups which had coefficient of similarities at 63%. Group A comprised J010703-11-1, E010206 (Korean pigs) and P6 (USA) which had similarity coefficients ranging from 65% to 90%. Group B was consisted of only one strain, P010726-26 (Korean cattle). Group C contained P1 and P2 (USA), and Group D comprised O157-R1-3-2 (Korean cattle). Group E showed 2 subgroups, E_1 and E_2. Subgroup E_1 included two isolates from Korean cattle, P010726-21 and P010726-24. Subgroup E_2 was broken down by 5 Korean isolates (P010726-18, P010726-22, P010726-23, P010726-25, and O157-C-1-2), and 14 USA isolates; 4 strains of ATCC (A1, A2, A3, and A4), 6 strains of Cornell University (C1, C2, C3, C4, C5, and C6), and 4 strains of Pennsylvania State University (P3, P4, P5, and P7). These strains in subgroup E_2 had a similarity coefficient of about 75%. Conclusively, 2 isolates from pigs in Korea had distinct genetic patterns from other strains. Three isolates from Korean cattle (P010726-18, 22, and 23) showed high similarity with USA isolates at 80% level. The USA isolates revealed close patterns with each other except three strains of Pennsylvania State University (P1, P2, and P6). Among three, P1 and P2 showed 70% similarity, and P6 revealed similar with two pig strains from Korea at 65% level. Six Korean strains from cattle showed coefficient of similarities from 63% to 80% level. The discriminatory power (*D*-value) of this RAPD fingerprinting assay was 0.86.

Vero cell cytotoxic assay

Cytotoxic effects of *E. coli* O157:H7 isolates were measured by Vero cell cytotoxic assay. CPE of eight isolates was strong, otherwise three was weak. The results of CPE of eleven *E. coli* O157:H7 isolates were shown in Table 5.

Antimicrobial susceptibility disc tests

A total of 23 antimicrobial discs were used in this study. Five of eleven *E. coli* O157:H7 isolates (45.5%) were resistant to two or more antimicrobial agents (Table 4). All isolates were resistant to erythromycin (100%) followed by

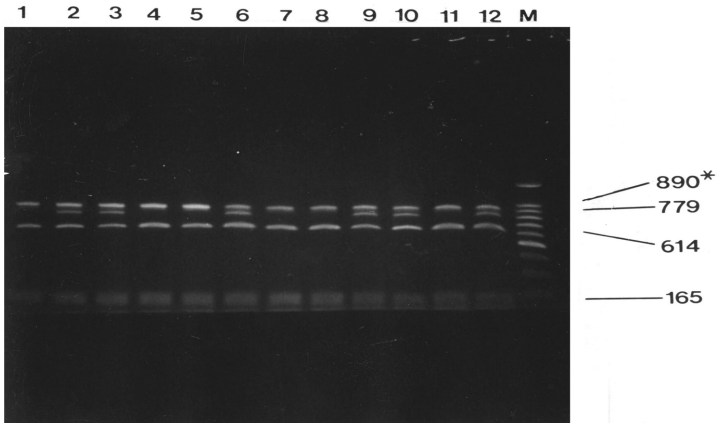

Fig. 2. Result of multiplex PCR assay for detection of the *Stx*1 (614 bp), *Stx*2 (779 bp), *eae*A (890 bp), and *hly*A (165 bp) genes in *E. coli* O157:H7 isolates. Lane 1, P010726-18; lane 2, P010726-21; lane 3, P010726-22; lane 4, P010726-23; lane 5, P010726-24; lane 6, P010726-25; lane 7, P010726-26; lane 8, E010206-13-2; lane 9, J010303-11-1; lane 10, O157-R1-3-2; lane 11, O157-C-1-2; lane 12, ATCC 43894 (a positive control); M, 100 bp DNA marker.

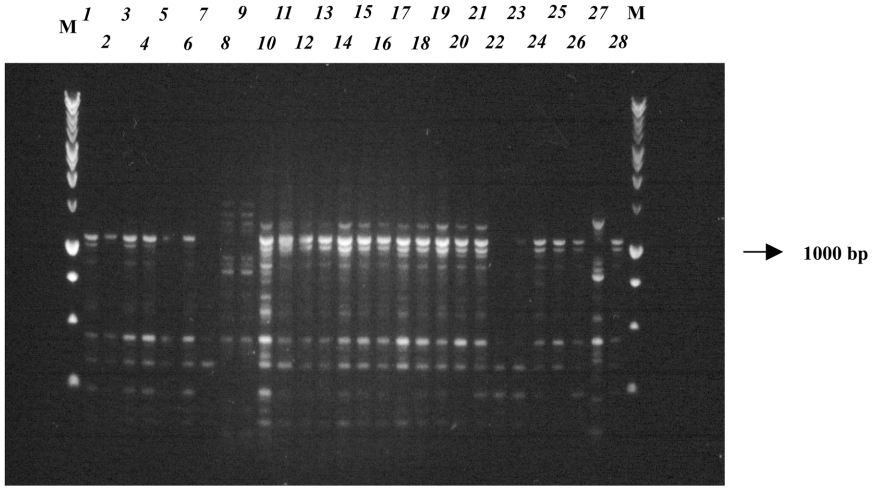

Fig. 3. RAPD patterns of 11 Korean isolates and 17 U.S. strains. Lane M, 1 kb DNA marker; lane 1, P010726-18; lane 2, P010726-21; lane 3, P010726-22; lane 4, P010726-23; lane 5, P010726-24; lane 6, P010726-25; lane 7, P010726-26; lane 8, E010206-13-2; lane 9, J010303-11-1; lane 10, O157-R1-3-2; lane 11, O157-C-1-2; lane 12, A1; lane 13, A2; lane 14, A3; lane 15, A4. lanes 16-21, strains C1, C2, C3, C4, C5, and C6, respectively (Cornell University strains); lanes 22-28, strains P1, P2, P3, P4, P5, P6, and P7, respectively (Pennsylvania State University strains).

ampicillin (27.2%), cephalothin (18.2%), and tetracycline (18.2%), respectively (Table 4).

Discussion

This study was conducted to determine the prevalence of STEC in cattle, pigs, and chickens using different detection methods and to define the molecular characteristics of the isolates using multiplex PCR and RAPD.

The conventional culture method showed the lowest detection rate. It might be attributable to lack of ability to detect *E. coli* O157:H7 which showed aberrant biochemical phenotypes [49]. In the case of IMS method, the detection rate was relatively high, however IMS was too labor-intensive when large numbers of samples were subjected to isolation [37]. The *E. coli* O157:H7 Rapid kit showed relatively high sensitivity and it only took 10 min to be proved to positive. Due to its sensitivity and rapidity, this would be useful to detect *E. coli* O157:H7 from various sources.

The detection rates of *E. coli* O157:H7 were variable among countries examined and detection methods they applied. The prevalence of *E. coli* O157:H7 from industrial minced beef was 0.12% in France [46], and other French researcher reported that there was no *E. coli* O157: H7 isolation in 1,200 samples [7]. In Switzerland, no *E. coli* O157:H7 was detected from 400 samples [15]. Five *E. coli* O157:H7 (3.3%) were isolated from retail beef and bovine

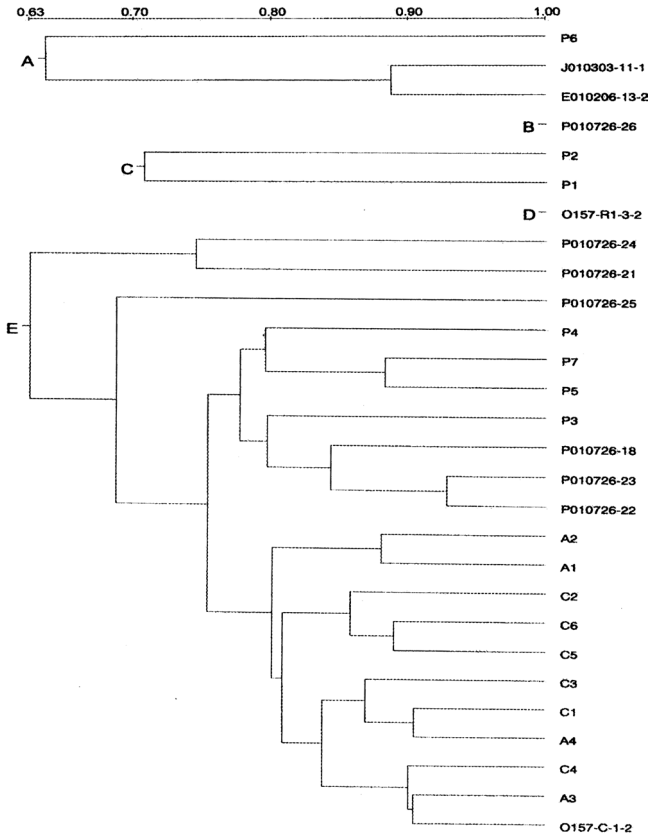

Fig. 4. The dendrogram constructed from RAPD data by UPGAMA method.

feces in Thailand, and 36 (8.7%) STEC in Spain [33]. The prevalence of STEC in North American and European cattle ranged from 0 to 10% [4]. The differences in the detection of STEC among these studies are probably due to the fact that the patterns of shedding of STEC are affected by diet, age, environmental condition, and seasonal variation [27]. The reasons of low detection rate in this study could be summarized into three factors. Firstly, limited sampling sources possibly influenced the detection rate [6,9]. Most sample sources (80%) in this work were obtained from bovine fecal and chicken rinsing samples. According to prevalence surveys about *E. coli* O157:H7 from domestic animals were less than 0.7% [6,9]. However, the proportions of STEC in calves and heifers were much higher than those in adults in other countries [12,21,33,41]. These authors demonstrated that young animals (calves and heifers) shed STEC more frequently than adults. In this study, most fecal samples were obtained from healthy adult cattle. Putting these studies together, age difference might be attributable to low detection rate of *E. coli* O157:H7 rather than sample sources. Secondly, seasonal variation might influence the detection rate in this study. Though the samples were collected all the year around, more samples were collected during January and February (38.3%). The rate of sampling from July to August was 20.2%. Many reports demonstrated that the distribution of *E. coli* O157:H7 was peaked between July and August [21,41]. The warmer and more moist conditions of the summer months may favor the survival and growth of STEC [21]. More sampling was conducted during summer season, more *E. coli* O157:H7 would be detected. Thirdly, most meat products were obtained from large-scaled retail markets which have relatively better hygiene conditions than small-scaled retail markets or meat shops [10,11].

According to H7 flagellar antiserum test and PCR, one isolate of Korean strains did not react in antiserum test. However, it showed positive at PCR assay for H7 gene. From this result, we could assume that the *E. coli* O157:H7 strain did not express its characteristic though they had H7 gene. Therefore, molecular determination by PCR should be performed to confirm.

We used RAPD fingerprinting assay to principally understand the molecular relatedness between the *E. coli* O157:H7 strains isolated from Korea and the USA. Since PFGE explores the whole length of chromosome whereas RAPD explores only randomly selected parts of it, RAPD analysis can be alternative method of PFGE typing method [36]. In general, high agreement between the results of the two methods was good for strain differentiation [25]. Moreover, Maurer *et al.* [28] claimed that fingerprinting by RAPD revealed more genetic differences among avian *E. coli* strains than restriction fragment length polymorphism (RFLP) analysis. Therefore, RAPD fingerprinting analysis was used for this study because its advantages of time and cost-saving, sensitivity, and no special skills required to perform.

The results of RAPD patterns in this study compared with the study of Radu *et al.* [36]. They reported 2 clusters and 22 isolates among 28 strains [36]. Of the 22 isolates, 3 predominant groups were observed and had 3 to 5 different bands. However, our study has revealed that the RAPD-PCR patterns were too diverse to differentiate the patterns of each *E. coli* O157:H7 isolates when the patterns were analyzed based on their band numbers. Using two primers CRA22 and CRA23 at least 7 bands were generated except 4 strains. Moreover, the discriminatory power (*D*-value) revealed 0.86. These diverse band patterns generated high *D*-value and differentiation among strains, so these two primers were recommended to dissect further molecular characteristics using RAPD analysis. At 63% similarity level, 5 clusters were generated by RAPD. Except B and D group, particularly E group showed that high genetic relatedness between strains at 75% level. Most USA strains had similar patterns except 3 Pennsylvania State University strains. More than 50% Korean cattle isolates were genetically similar to the USA cattle isolates. However, the reason that distinct genetic pattern between pig and cattle isolates from Korea may depend on their species difference of sources.

Several studies demonstrated that source differentiation could be determined by RAPD [32,35]. Therefore, this technique could be of use when studying the epidemiology of *E. coli* O157:H7. Although RAPD had a capacity to distinguish strains with different virulence factors from different sources, we could not define the difference in the genetic patterns between strains possessing only *stx*1 or *stx*2 and strains possessing both *stx*1 and *stx*2. RAPD has revealed that it could not discriminate among isolates according to their differences either in the degree of virulence in several studies [8,30].

E. coli O157:H7 was reportedly susceptible to many antibiotics [42]. Approximately 45.5% of the present strains showed antibiotic resistance to two or more of the antimicrobial agents used in this study. Their antibiotic resistance was against erythromycin (100%), followed by ampicillin (27.2%), cephalothin (18.2%), and tetracycline (18.2%). Antimicrobial resistance patterns were observed most commonly to ampicillin (25.4%), tetracycline (23.8%), and streptomycin (14.3%) and less frequently to cephalothin (11,1%) and nalidixic acid (6.4%) in India [25]. The USA study about antibiotic resistance showed that all isolates were resistant to tilmicosin, and most isolates were susceptible to trimethoprim/sulfamethoxazole and ciprofoloxacin [17]. In Malaysia, resistance was observed mostly towards bacitracin (100%), sulphafurazole (77%), ampicillin (57%), cephalothin (53%), and carbenicillin (30%) [36]. The antibiotic resistant patterns to ampicillin, fosfomycin, kanamycin, and vancomycin were observed in Japan [45]. From these data, *E. coli* O157:H7 was mainly resistant to ampicillin and tetracycline. Resistance patterns of Korean isolates were similar to those of Malaysian. The possibility of the change of resistance patterns could not exclude the percentage of intermediately resistant group which revealed relatively high to carbenicillin (72.7%), ampicillin (54.5%), amoxicillin/clavulanic acid (45.5%), kanamycin (27.3%), polymyxin B (36.4%), streptomycin (36.4%), tetracycline (36.4%), and cephalothin (27.3%).

This study has found that the prevalence of *E. coli* O157:H7 was not as high as that of other countries. However, the *E. coli* O157:H7 has been isolated from various livestock processing stages from slaughtering to processing. Therefore, more careful investigation programs such as HACCP should be applied to establish all dairy herds, slaughterhouses, and meat processing plants. The *E. coli* O157:H7 Rapid kit which examined in this study was apparently useful to detect the contamination of *E. coli* O157:H7 with high accuracy and rapidity. In addition, RAPD results indicated that Korean cattle isolates were genetically related with those of the USA strains at 70% similarity level, which could assume similar mechanism of contamination in animals and related sources. Continuous monitoring and surveillance program for examining microbial contamination of imported feeds should be performed to minimize the risk of spread of major food-borne pathogens.

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