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Escherichia coli O157:H7 is recognized as an important food-borne pathogen, being implicated in over 2,500 deaths worldwide. Various genetic typing methods have been investigated in studying possible outbreak strains, including pulsed-field gel electrophoresis (PFGE), randomly amplified polymorphic DNA (RAPD), and enterobacterial repetitive intergenic consensus polymerase chain reaction (ERIC-PCR). These genetic methods yield information on the genetic diversity of organisms, but make no attempt to identify specific virulence genes. In this study, we present a modified RAPD which targets specific virulence genes under low stringency conditions and also yields information on the diversity of the organism in question. Plasmid profiling, ERIC-I PCR, and RAPD were also used to determine genetic heterogeneity for comparison. Our results indicate the modified RAPD may be a better tool for investigating the genetic relationships of organisms.

Genetic variability of *Escherichia coli* O157:H7

A Thesis

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Preface

This thesis was prepared following the publication style of American Society for Microbiology.

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BACKGROUND REVIEW

I. *Escherichia coli*

Escherichia coli is a gram-negative, rod-shaped organism that belongs to the family *Enterobacteriaceae*. *E. coli* strains which cause adult and infant gastroenteritis, can be distinguished into several distinct types. These types include entrohemorrhagic (EHEC), enteropathogenic (EPEC), enterotoxigenic (ETEC), enteroinvasive (EIEC), enteroaggregative (EaggEC), uropathogenic (UPEC), and neonatal meningitis (MNEC) *E. coli* (26). Surface constituents, such as heat-stable somatic (O) antigens, heat-labile somatic (K) antigens, and heat-labile flagellar (H) antigens, are used to describe the different serotypes of *E. coli*. Currently, 173 O antigens, 103 K antigens, and 56 H antigens have been described (24, 26).

II. Pathogenesis of *Escherichia coli* O157:H7

Escherichia coli O157:H7 was first identified as a food-borne pathogen in 1975 and has escalated as a significant pathogen causing morbidity and mortality worldwide (5, 50). Since 1975, approximately thirty major outbreaks have been reported worldwide (13, 14, 19). In the United States, *E. coli* O157:H7 infection was first

reported in 1982 in the state of Washington (12, 19). It is estimated that *E. coli* O157:H7 infects approximately 10,000 to 20,000 people worldwide annually, with approximately 250 deaths (13).

Consumption of contaminated undercooked ground beef contributes to 40% of infections; consumption of contaminated raw milk, apple cider, venison jerky, dry-cured salami, vegetables, salad dressing, water, and person-to-person contact contribute to the majority of the remaining cases (14, 13, 17, 19). Generally, food with pH values lower than 4.6 are considered at low risk for *E. coli* O157:H7. This organism can however, survive at pH values as low as 2.0 (14, 19). Approximately 0.3% to 2.2% of healthy cattle carry *E. coli* O157:H7 in their gastrointestinal tract and when shed into feces, it can survive for up to 70 days (12, 17).

1. Virulence factors

Shiga-like toxins (SLT), also known as verotoxin (encoded by *stx*), hemolysin (encoded by *hly*), and intimin (encoded by *eae*) are the major virulence factors that contribute to diseases caused by *E. coli* O157:H7 (2, 9, 23, 27, 28, 37, 39, 48, 57). *E. coli* O157:H7 can be distinguished into EHEC, EPEC, and ETEC. These three

groups of *E. coli* O157:H7 share a similar model of pathogenesis, which includes an attaching and effacing lesion (42). If *E. coli* O157:H7 produces SLT, it is characterized as EHEC. ETEC are characterized by no SLT production, whereas EPEC is defined by the production of intimin (42).

a.) Shiga-Like Toxins (SLT)

The O157:H7 strains that produce shiga-like toxin are the most virulent strains of *E. coli*. There are two forms of SLT, referred to as SLT-I and SLT-2, with SLT-2 being the most prevalent (5, 19, 22, 23, 25, 27, 37, 39). SLT-1 and SLT-2 are both 68 kDa proteins which are composed of one A subunit (~ 32 kDa), and five B subunits (~ 7.7 kDa) encoded by *stx* (37). SLT-1 is so named because it cross reacts with shiga toxin produced by *Shigella dysenteriae*; however, SLT-2 does not cross react (5, 22, 23, 28, 37). SLT inhibits protein synthesis through an enzymatic modification by removing a purine from the 28S RNA which prevents elongation during translation (25, 37). Also, SLT can damage vascular endothelial cells by binding to glycosphingolipid (Gb3) in the plasma membrane, thereby inhibiting the phosphatidylinositol pathway (25, 28, 37, 52).

b.) Intimin

Intimin is a 97 kDa protein encoded by *eae*, a component of the locus for enterocyte efffacement operon (LEE) (7, 9, 21, 22, 28, 47). Intimin causes polymerization of actin through a localized cytoskeleton rearrangement at the junction where the organism attaches to a cellular surface. This results a cup-like pedestal structure known as an attaching and effacing (A/E) lesion (2, 9, 57). Studies have also indicated intimin might alter the blood barrier and increase the permeability of SLT (37).

c.) Hemolysin

Three different hemolysins, know as α-hemolysin (encoded by *hly* and *ely*), β-hemolysin, and enterohemolysin (encoded by *ehx*) are associated with *E. coli* O157:H7 (31, 33, 49). The two different α-hemolysins produced by *E. coli* O157:H7 are related to the RTX family of toxins. RTX toxins are pore-forming proteins that have repeats in their structure (1, 33, 49). The most common α-hemolysin is encoded by *hly*, whereas little is known about the products of *ely* (22, 48). The α-hemolysin encoded by *hly* is part of an operon that encodes five genes (*hly*A, B, D,

E, and *tol*C) (10, 31, 48, 49). The gene product of *hly*A is a 110 kDa protein that causes lysis of erythrocytes (48, 49). Enterohemolysin, encoded by *ehx*, is strongly associated with the production of SLT in EHEC. It is produced only in the late phase of growth, but its mechanism of action remains to be elucidated (4, 45). β- hemolysin has a similar cytocidal effect as α- hemolysin, but little is known about the mechanism of action of this toxin (4).

2. Diseases caused by *E. coli* O157:H7

Various symptoms are associated with *E. coli* O157:H7 infection including diarrhea, bloody diarrhea, vomiting, abdominal cramps, and sometimes no symptoms at all (5, 12, 13, 19, 35). The wide range of symptoms caused by this organism is due to phenotypic variation. For example, patients with EPEC infections usually have no or very mild symptoms; however, infection with EHEC results in more severe symptoms such as acute renal failure, encephalopathy, hemorrhagic colitis (HC), and microangiopathic thrombocytopenic disorders including hemolytic-uremic symptom (HUS) and thrombotic thrombocytopenic purpura (13, 23, 25, 27, 37, 39, 42, 48).

3. Clinical Identification

Sorbitol fermentation tests, including cefixime-tellurite sorbital MacConkey agar and sorbitol MacConkey agar, are widely used for identification of *E. coli* O157:H7 (19). *E. coli* O157:H7 has no sorbitol fermentation in 24 hour and has a negative 4-methylumbelliferyl-B-D-glucuronide (MUG) test (19). The use of hemorrhagic colitis agar and anti-O157 antibody is also used to confirm the identification of *E. coli* O157:H7 (17, 19).

III. Bacterial Subtyping

Different methods for bacterial subtyping have been used to identify different strains within species for taxonomic or epidemiological studies. The purpose of subtyping is to distinguish epidemiologically related strains and to show the origination of the strains (6). Ideally, the bacterial subtyping system must be fast, reproducible, highly sensitive, and able to distinguish outbreak strains from non-outbreak strains (6, 32). The three major subtyping systems that are used to distinguish different strains are serotyping, biotyping, and molecular typing methods. Currently, molecular typing methods are the most commonly used. Numerous molecular typing methods

have been described, including pulsed field gel electrophoresis (PFGE), ribotyping, restriction fragment length polymorphism (RFLP), multilocus enzyme electrophoresis (MLEE), polymerase chain reaction (PCR) based approaches, plasmid profiling, phagetyping, low molecular weight rRNA profiling, and Southern Blot (5, 6, 10, 13, 14, 24, 26, 39, 41, 45, 48, 50, 52). Each has its advantages and limitations.

1. Pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis (PFGE) is considered to be reproducible, highly sensitive, and is widely accepted for epidemiological typing of bacteria (6, 14, 17). Genomic DNA is isolated and digested with a restriction endonuclease. A restriction map is obtained by electrophoresis using a clamped homogeneous electric field with low voltage for a long period of time. DNA banding patterns are compared to determine the relationship between each isolate (28).

2. Ribotyping

Ribotyping analyzes restriction polymorphisms within the ribosomal RNA gene. Isolated bacterial DNA is digested with various endonucleases and DNA fragments separated by electrophoresis. The separated DNA is transferred to a

nylon membrane and hybridized with a probe consisting of the 16S rRNA or 23S rRNA gene fragment (18, 16, 43, 51). The gene sequences of the 16S rRNA and 23S rRNA are species specific and it is possible to differentiate organisms (43). A PCR based ribotyping approach is also available which amplifies 16S rRNA or 23S rRNA genes (15, 18, 55).

3. Restriction fragment length polymorphism

Restriction fragment length polymorphisms (RFLP) are generated once DNA is digested with various restriction endonucleases and separated by electrophesis (13, 15, 4[illegible].

Alternatively, this method can be accomplished using a PCR-based approach, designated amplified restriction fragment length polymorphism (AFLP) (15).

4. Repetitive sequence based PCR

Repetitive sequences exist in intergenic regions, polycistronic operons, and untranslated regions in the genome of organisms. Though the functions of these regions are unclear, they appear to play a role in the regulation of transcriptional termination, protein binding, and chromosomal maintenance (32, 38, 44). Repetitive intergenic consensus sequences are highly conserved among gram-negative organisms (8). Different types of PCR that target specific repeating elements have been used to

cluster organisms genotypically (20, 29, 30, 32, 36, 54). Enterobacterial repetitive intergenic consensus sequence-PCR (ERIC-PCR) is commonly used in the identification of the *Enterobacteriaceae* family and related phyla (32). This approach targets a 126 base pair repetitive element under high stringency conditions (6, 20, 45).

5. Randomly amplified polymorphic DNA

Randomly amplified polymorphic DNA PCR (RAPD) uses a single, short, arbitrary primer under low stringency conditions to generate strain-specific information (24, 53, 55). With this method, no prior sequence information is required; however, it is compromised by low levels of reproducibility (40, 50, 55). In recent years, RAPDs have been used in the identification, molecular typing and genetic characterization of various organisms, such as *Aspergillus fumigatus*, *E. coli* O157:H7, *Vibrio vulnifuis*, *Neagleria fowleri*, *Blastomycres dermatitidis*, *Borrelia burgdorferi*, and *Neisseria meningitis* (1, 7, 24, 12, 15, 23, 48, 50, 55).

6. Other PCR approaches

Many distinct PCR based methods have found their utility in the identification and genetic characterization of organisms. Some of the most popular methodologies

include amplified rDNA restriction analysis (ARDRA), multiplex PCR, polymorphic G-C rich repetitive PCR, and ribosomal intergenic spacer sequence PCR amplification (6, 13, 15, 39, 41, 48).

7. Plasmid profile and phagetyping

Plasmids of bacteria are isolated and analyzed by agarose gel electrophoresis to determine the sizes present, thereby producing information on genetic heterogeneity (34). Phagetyping can also be useful to determine genetic variation and tests the susceptibility of the organism in question to a bacteriophage or bacteriocin (34).

METHODS AND MATERIALS

I. Bacterial strains and growth conditions

Fifty-seven *Escherichia coli* O157:H7 strains, isolated from beef feed yards in southwest Kansas, were obtained from the Food Animal Health and Management Center, College of Veterinary Medicine, Kansas State University, Manhattan, Kansas. As reference strains, *E. coli* O157:H7 SLT-I and SLT-II negative (ATCC 43888), SLT-I and SLT-II positive (ATCC 43894), SLT-I positive only (ATCC 43890), and SLT-II positive only (ATCC 43889), were obtained from the American Type Culture Collection (Manassas, VA). All *E. coli* O157:H7 strains were routinely propagated in Brain Heart Infusion (BHI) media or on agar plates at 37 C. A complete guide of media components and solutions used in this study are shown in Table 1. Chemicals were obtained from either Fisher Scientific (St. Louis, MO) or Sigma (St. Louis, MO).

II. Chromosomal DNA isolation

Chromosomal DNA was isolated using a modification of an established method (47). Bacterial cultures were inoculated into 6 mL of BHI broth contained in 20 mL test tubes and grown approximately 18 hours at 37°C with shaking at 250 rpm in a New Brunswick series 25 incubator shaker (Edison, NJ). Cultures were placed into 15 mL conical

centrifuge tubes and cells harvested by centrifugation at 4,000 rpm for 5 min in a model GLC-1 Sorvall tabletop centrifuge (Newtown, CT). The supernatant was discarded and cells resuspended in 2 mL of Tris-EDTA (TE) buffer. One hundred microliters of 20% sodium dodecyl sulfate (SDS) was added, and the solution gently mixed by inversion. After incubation at 60°C for 15 min, 20 µL of RNase (10 mg/mL) was added and the solution incubated at 37°C for 20 min. Contaminating proteins were digested upon addition of 15 µL proteinase K (10 mg/mL) and subsequent incubation at 60°C for 1 hour. Phenol extraction was performed by adding 2 mL of equilibrated phenol to the cellular lysate and the solution mixed by inversion gently for 2 min. After centrifugation at 4,000 rpm for 5 min, the aqueous phase was transferred into a clean 15 mL conical tube. Additional phenol and chloroform/isoamyl alcohol (24:1) extractions were repeated as described above. The aqueous phase, after the final extraction, was transferred into a clean 15 mL conical centrifuge tube and 10 mL of ice-cold 95% ethanol was added. The solution was incubated at -20°C for 10 min to precipitate chromosomal DNA. Precipitated DNA was removed using a glass Pasteur pipette and

transferred into a 1.5 mL microcentrifuge tube. After drying in a Savant Vacuum model IS550 speed dryer (Holbrook, NY) for 2 to 5 min at high setting, the DNA was resuspended in 200 μL of TE buffer. Analysis of the DNA solution was performed at 260 and 280 nm to determine the DNA concentration and purity. DNA solutions were stored at 4°C until needed.

III. Quantification of DNA

DNA concentration was determined using a Beckman model DU 530 spectrophotometer (Palo Alto, CA). For analysis, DNA containing solutions were diluted 200 fold with sterilized TE buffer. One milliliter of the diluted DNA solution was placed in a 1 mL quartz cuvette and the absorbance measured at 260 and 280 nm. The concentration of DNA solutions was calculated using the following equation (47):

DNA concentration (μg/mL) = 260 nm reading × dilution factor × 50 μg/mL

DNA Purity = Ratio of 260/ 280 nm reading

IV. Plasmid DNA isolation

Plasmid DNA isolation was performed using a modification of an established method (47). Three milliliter bacterial cultures were grown approximately 18 hours in LB containing the appropriate antibiotic at 37°C with shaking at 250 rpm. Half of the culture was placed in a 1.5 mL microcentrifuge tube and cells collected by centrifugation at 13,000 rpm for 30 seconds. The supernatant was discarded, the remaining culture added, and the process of centrifugation repeated. Cells were resuspended in 200 μL of solution I. Cell lysis was accomplished upon addition of 200 μL of solution II, followed by incubation at room temperature for 5 to 10 min. One hundred and fifty microliters of solution III was added and the solution mixed gently by inversion. After incubation on ice for 10 min, centrifugation at 13,000 rpm for 10 min was performed. The supernatant was transferred to a clean 1.5 mL microcentrifuge tube, and 10 μL of RNase (10 mg/mL) added. After incubation at room temperature for 10 min, 500 μL of 1.6 M NaCl-13% polyethylene glycol [illegible] was added to precipitate plasmid DNA. Precipitated plasmid DNA was collected by centrifugation at 13,000 rpm for

10 min, and resuspended in 400 μL TE buffer. One phenol and one chloroform/isoamyl alcohol extraction were performed as described for chromosomal DNA isolation. Plasmid DNA was precipitated upon addition of 1 mL of 95% ethanol and collected by centrifugation at 13,000 rpm. Isolated plasmid DNA was resuspended in 20 μL of TE buffer, quantified as described above, and stored at 4°C.

V. Polymerase Chain Reaction

Amplification of DNA was carried out by the polymerase chain reaction (PCR) using a model PT150 Minicycler (MJ Research Inc. Watertown, MA). All reactions contained template DNA (500 ng) and 100 nmol of each primer in a reaction buffer consisting of 10 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each deoxynucleotide triphosphate (dNTPs), and 1 U of Taq polymerase in a final volume of 100 μL. A typical amplification consisted of an initial denaturation step at 94°C for 5 min, followed by 30 cycles of DNA denaturaion at 94°C for 1 min, annealing (time and temperature variable), and elongation at 72°C (time variable). Specific primers used and PCR conditions are listed in Table 2.

VI. Agarose gel electrophoresis

Agarose gel electrophoresis was used to separate DNA fragments according to size (47). Tris-Acetate-EDTA (TAE) was the buffer system used in preparing the gel and running buffer. A guide to preparing the various sized gels is shown in Table 3. Agarose gels were analyzed with a model FBTIV-88 UV transilluminator (Fisher Scientific) at 254 nm and documented by an Ultra-Lum Panasonic model CCD CAMERA photodocumentation system (Paramount, CA).

VII. Generation of Dendogram

Agarose gels were analyzed visually and each isolate was scored in terms of its unique banding pattern. Data were transferred into binary code using Microsoft EXCEL. Binary data were analyzed by SPSS Windows release 8.0.0 to perform the Euclidean distance coefficient test. Based on the Euclidean distance coefficient, the unweighted pair group method average (UPGMA) was used to generate the dendogram.

Table 1. Growth media and solutions used in this study

Name	Ingredients
Luria-Bertani (1L)	10 g NaCl 5 g Yeast Extract (Difco) 10 g Tryptone (Difco)
BHI (1L)	37 g BHI media (Difco)
TE buffer (pH 8.0)	10 mM Tris 1mM EDTA
Solution I	50 mM Sucrose 25 mM Tris 10 mM EDTA
Solution II	0.2 N NaOH 1% SDS
Solution III (100 mL)	3 M Potassium acetate 11.5 mL Acetic acid
$CaCl_2$ media	0.1 M $CaCl_2$ 0.1 M $MnCl_2$ 0.04 M Sodium acetate
50x TAE buffer	242 g Tris Galacial acetic acid 57.1 mL 0.5 M EDTA 4 mL Added water to 1L
1x TAE buffer (1L)	20 mL 50x TAE buffer

Table 2. Primers and reaction conditions used in PCR

Primer	Primer sequence 5′-3′	Size of product (bps)	Conditions
stx I	Forward: ATAAATCGCGCAACAAGAGG Reverse: GGCACGCCCACTGAGATCATC	180	94 °C 1 min 57 °C 2 min 72 °C 30 sec
stx II	Forward: GGCACTGTCTGAAACTGCTCC Reverse: TCGCCAGTTATCTGACATTCTG	255	94 °C 1 min 55 °C 2 min 72 °C 30 sec
eaeA	Forward: GACCCGGCACAAGCATAAGC Reverse: CCACCTGCAGCAACAAGAGG	384	94 °C 1 min 55 °C 2 min 72 °C 30 sec
hlyA	Forward: GCATCATCAAGCGTACGTTCC Reverse: AATGAGCCAAGCTGGTTAAGG	650	94 °C 1 min 55 °C 2 min 72 °C 30 sec
ERIC-I	GTGAATCCCCAGGAGCTTACAT	Variable	94 °C 1 min 52 °C 2 min 72 °C 2min
1283	GCGATCCCCA	Variable	94 °C 1 min 55 °C 1 min 72 °C 30 sec
1254	CCGCAGCCAA	Variable	94 °C 1 min 55 °C 1 min 72 °C 30 sec

Table 3. Preparation of agarose gels and electrophoresis conditions

Types of gel	Ingredients	Running conditions
0.7 % 30 mL gel	30 mL of 1xTAE buffer 0.21 gram Agarose 1 μL 10 mg/mL Ethidium bromide	Voltage: 95 V Time: 1.5 hr Running buffer: 250 mL TAE buffer
1.5 % 30 mL gel	30 mL 1x TAE buffer 0.45 g Agarose 1 μL 10 mg/mL Ethidium bromide	Voltage: 95 V Time: 1.5 hr Running buffer: 250 mL TAE buffer
0.7 % 150 mL gel	150 mL 1x TAE buffer 1.05 g Agarose 5 μL 10 mg/mL Ethidium bromide	Voltage: 115 V Time: 1.5 hr Running buffer: 1.75 L TAE buffer
1.5 % 200 mL gel	200 mL 1x TAE buffer 3 g Agarose 6 μL 10 mg/mL Ethidium bromide	Voltage: 115 V Time: 1.5 hr Running buffer: 1.75 L TAE buffer

RESULTS

Total DNA from 57 *E. coli* O157:H7 isolates and *E. coli* O157:H7 reference strains were isolated. The concentration and purity of the isolated DNA were determined by measuring the absorbance at 260 and 280 nm. The results are listed in Table 4. Each sample was standardized to 10 µg/mL for use in PCR. All isolates were tested for the existence of virulence genes and the results listed in Table 5.

I. Characterization of *E. coli* O157:H7 isolates using *stx1* and *stx2* primers

Primers designed to target the SLT genes were used to identify the genes and determine genetic variation. The *stx1* primer pair (Table 2) was designed to amplify a 348 base pair (bp) region within the A subunit of SLT-1. When primers were annealed at 57°C to template DNA, 34 out of the 57 isolates, representing 57.89% of the sample population, were positive for *stx1* (Figure 1A). A total of 171 bands and 17 unique banding patterns was observed after agarose gel electrophoresis. These results were transferred into binary code to determine distance coefficients. Based on the calculated distance coefficients and UPGMA, a dendogram was generated (Figure 1B). A total of 34 different groups

with unique amplification patterns was observed. On the contrary, primer *stx2* (Table 2) designed to amplify a 255 bp fragment within the A subunit of SLT-2, amplified this fragment in 38 out of the 57 *E. coli* O157:H7 isolates (64.91%), test results are listed in Table 5. A total of 112 bands and 14 unique banding patterns was observed after agarose gel electrophoresis (Figure 2A). Dendogram analysis yielded 14 genotypic patterns (Figure 2B).

II. Characterization of *E. coli* O157:H7 isolates using *hlyA* primers

Oligonucleotides targeted at *hlyA* were used to identify a 534 bp fragment of *hly* and determine the genetic variability of the *E. coli* O157:H7 isolates. Primers based on *hlyA* (Table 2) were annealed at 55°C, and 24 of the 57 *E. coli* O157:H7 isolates (Table 5), representing 42.11% of the sample population, were positive for the 534 bp band as determined by agarose gel electrophoresis (Figure 3A). A total of 58 bands and 8 unique banding patterns was observed. Dendogram analysis based on these data revealed 15 groups (Figure 3B).

III. Characterization of *E. coli* O157:H7 isolates using *eaeA* primers

Primers targeted at *eaeA* (Table 2) were used to identify a 384 bp internal fragment of the *eaeA* gene and generate genotypic information on the 57 *E. coli* O157:H7 isolates. Twenty-six out of 57 samples (45.61%) were positive for the expected 384 bp band as determined by agarose gel electrophoresis (Figure 4A). A total of 226 bands and 9 unique banding patterns was analyzed to generate the dendogram profile shown in Figure 4B. Based on dendogram analysis, 25 groups were revealed.

IV. Characterization of *E. coli* O157:H7 isolates using ERIC-1 primer

A primer designed to target enterobacterial repetitive intergenic consensus sequences was used in a PCR. The sequence of this primer is shown in Table 2. Agarose gel electrophoresis revealed a total the 153 bands and 17 unique banding patterns (Figure 5A), resulting in 22 groups based on dendogram analysis (Figure 5B).

V. Characterization of *E. coli* O157:H7 isolates using RAPD profiles

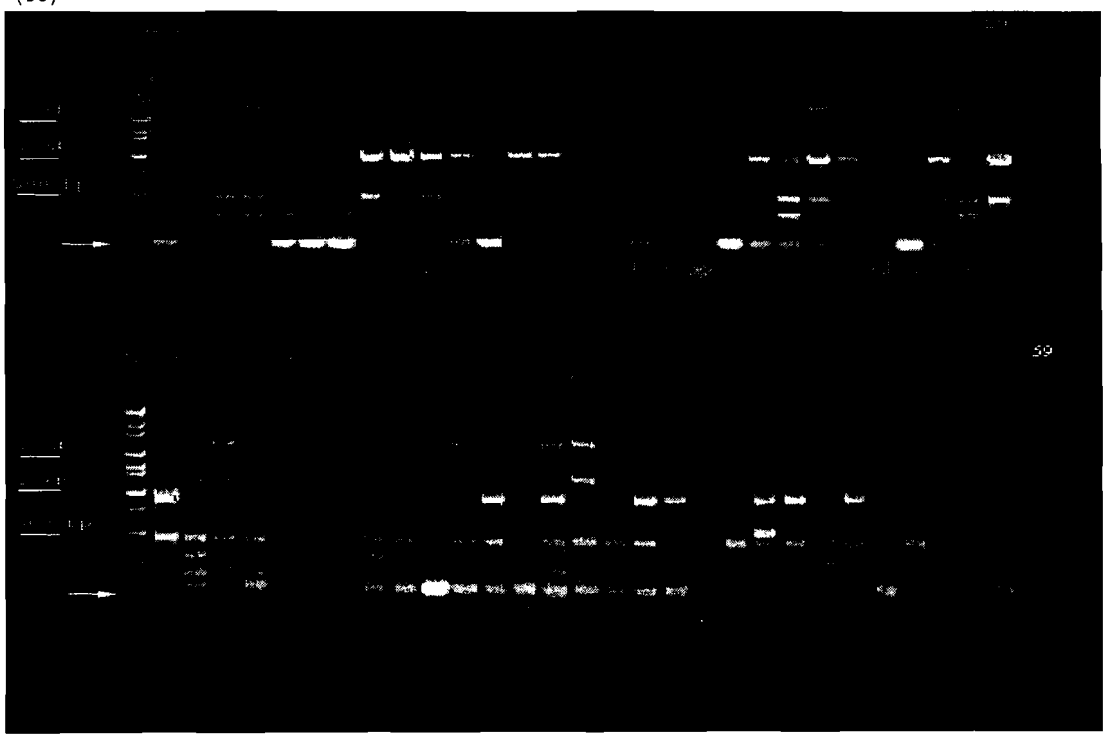
Two arbitrary primers, designated 1254 and 1283 (Table 2), were used in RAPD analysis of the 57 *E. coli* O157:H7 isolates. Primer 1254 generated 394 bands and 27 unique banding patterns as determined by agarose gel electrophoresis (Figure 6A), whereas primer 1283 yielded 448 bands and 30 unique banding patterns (Figure 7A). Dendogram profiles were generated using these data. Primer 1254 yielded 46 genotypic groups (Figure 6B), whereas primer 1283 yielded 46 groupings (Figure 7B).

VI. Characterization of *E. coli* O157:H7 isolates using plasmid profiles

Plasmids from the 57 *E. coli* O157:H7 isolates were isolated and analyzed by agarose gel electrophoresis (Figure 8A). A total of 153 bands and 10 unique banding patterns were observed. Dendogram analysis revealed 19 groups (Figure 8B).

Fig. 1. Characterization of *E. coli* O157:H7 isolates using *stx1* primers. (A) 1.5% agarose gel of amplified reaction mixtures. Lane M1 and M2: DNA markers; Lane 1 to 57: *E. coli* O157:H7 feedyard isolates; Lane 58, *E. coil* O157:H7 ATCC 43888; Lane 59: *E. coli* O157:H7 ATCC 43894. The arrow indicates the 180 bp *stx1* amplification product. A total of 171 bands and 17 unique banding patterns was observed after agarose gel electrophoresis. (B) Dendogram of *E. coli* O157:H7 isolates and ATCC reference strains. Dendogram analysis yielded 34 genotypic patterns. The groupings are designated A to AH.

(A)



(B)

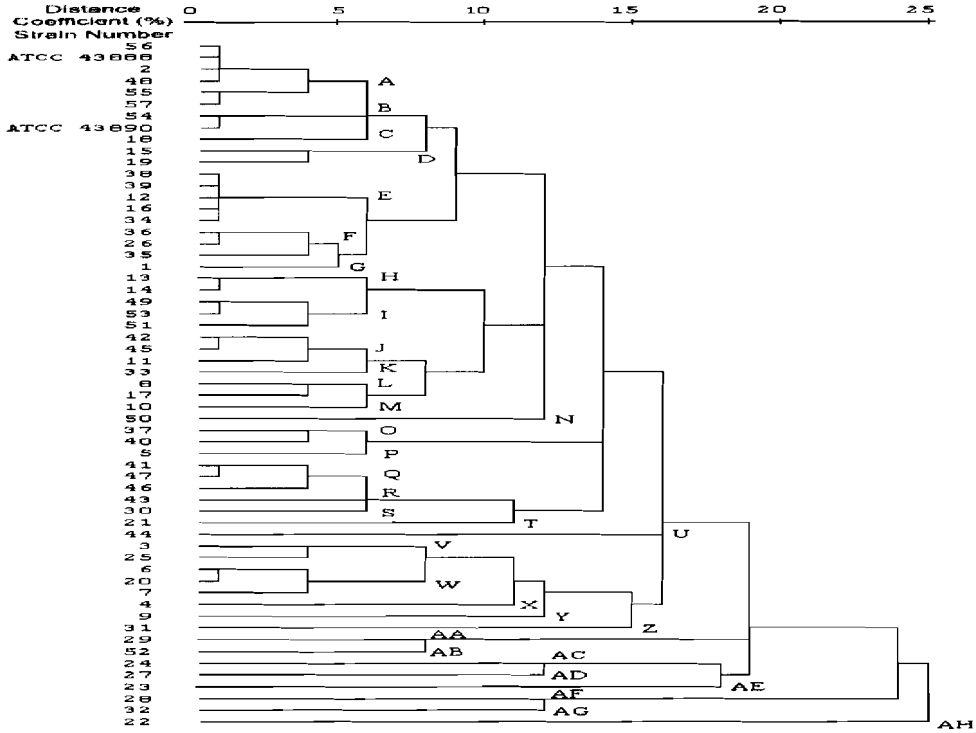
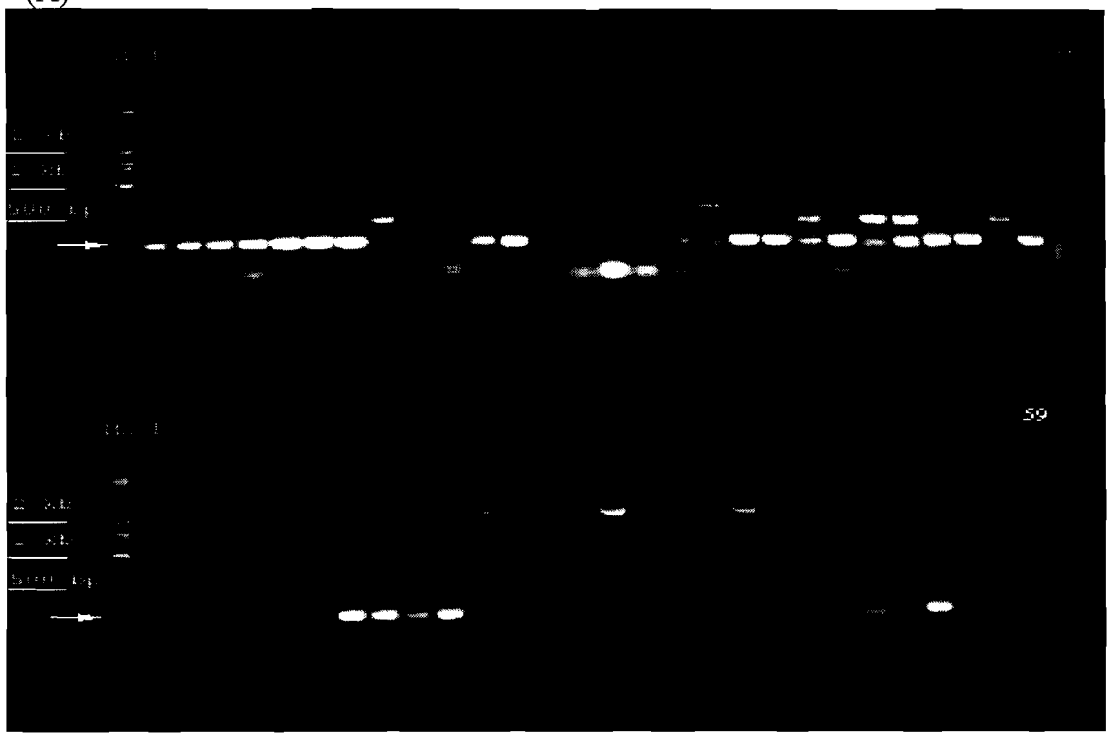
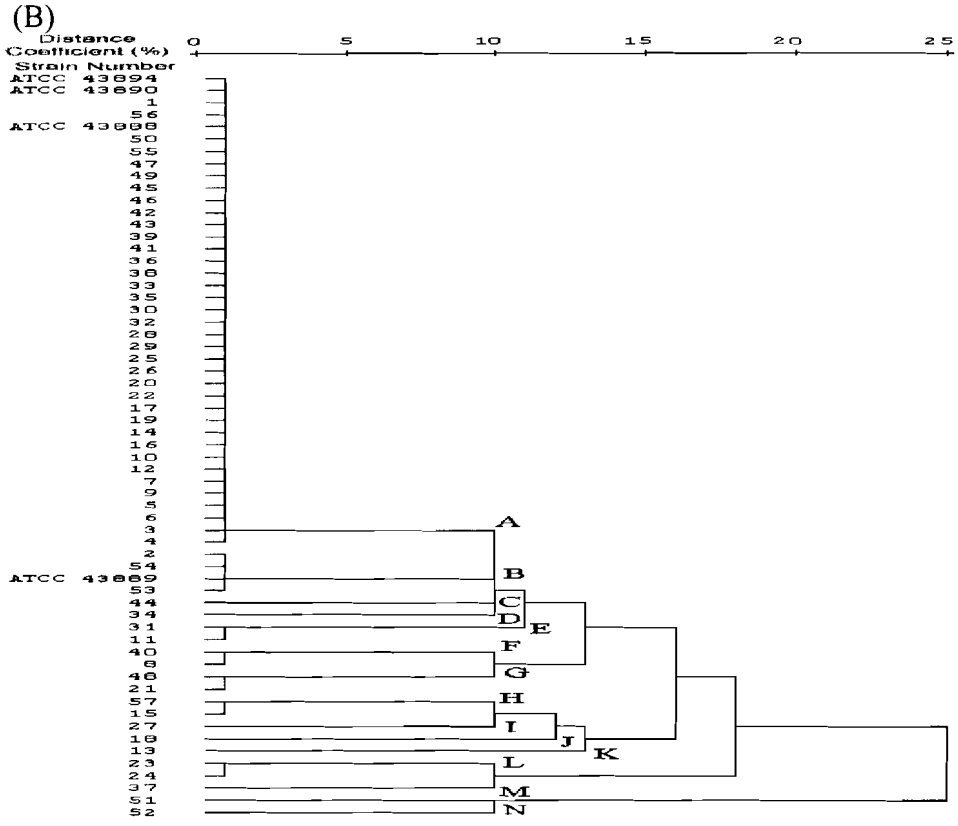

Distance
Coefficient (%)
Strain Number
0
5
10
15
20
25
ATCC 43888
ATCC 43890
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T
U
V
W
X
Y
Z
AA
AB
AC
AD
AE
AF
AG
AH

Fig. 2. Characterization of *E. coli* O157:H7 isolates using *stx2* primers. (A) 1.5% agarose gel of amplified reaction mixtures. Lane M1 and M2: DNA markers; Lane 1 to 57: *E. coli* O157:H7 isolates; Lane 58, *E. coil* O157:H7 ATCC 43888; Lane 59: *E. coli* O157:H7 ATCC 43894. The arrow indicates the 255 bp *stx2* amplification product. A total of 112 bands and 14 unique banding patterns was observed after agarose gel electrophoresis. (B) Dendogram of *E. coli* O157:H7 isolates and ATCC reference strains. Dendogram analysis yielded 14 genotypic patterns. The groupings are designated A to N.

(A)



(B)


Distance
Coefficient (%)
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10
15
20
25
Strain Number
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ATCC 43890
1
56
ATCC 43888
50
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9
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6
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4
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ATCC 43889
53
44
34
31
11
40
8
48
21
57
15
27
18
13
23
24
37
51
52
A
B
C
D
E
F
G
H
I
J
K
L
M
N

Fig. 3. Characterization of *E. coli* O157:H7 isolates using *hlyA* primers. (A) 1.5% agarose gel of amplified reaction mixtures. Lane M1 and M2: DNA markers; Lane 1 to 57: *E. coli* O157:H7 isolates; Lane 58, *E. coil* O157:H7 ATCC 43888; Lane 59: *E. coli* O157:H7 ATCC 43889; Lane 60: *E. coli* O157:H7 ATCC 43894, Lane 61, *E. coli* O157:H7 ATCC 43890. The arrow indicates 650 bp *hlyA* amplification product. A total of 58 bands and 8 unique banding patterns was observed. (B) Dendogram of *E. coli* O157:H7 isolates and ATCC reference strains. Dendogram analysis yielded 15 genotypic patterns. The groupings are designated A to O.

(A)

(B)

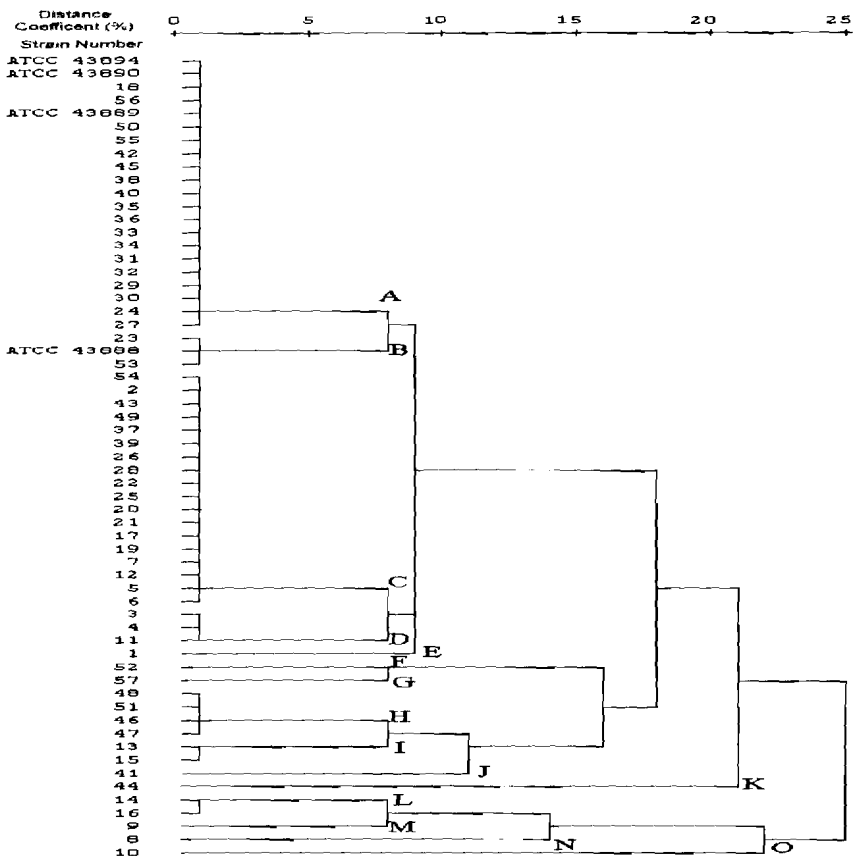
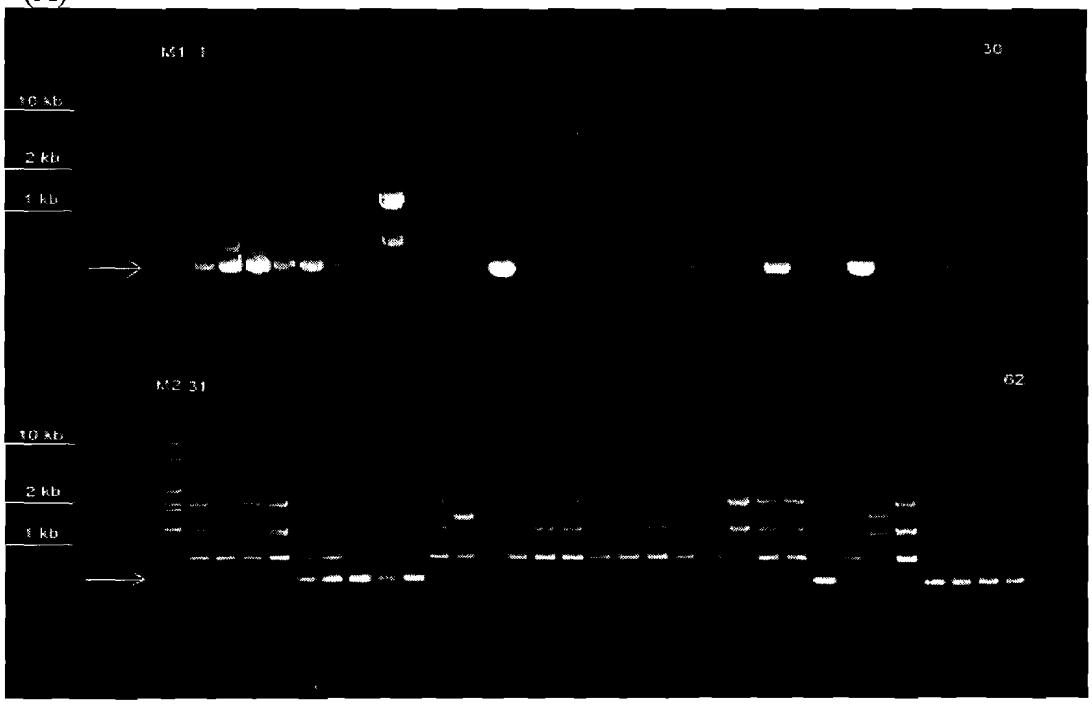

Distance
Coefficent (%)
Strain Number
0
5
10
15
20
25
ATCC 43894
ATCC 43890
18
56
ATCC 43889
50
55
42
45
38
40
35
36
33
34
31
32
29
30
24
27
23
ATCC 43888
53
54
2
43
49
37
39
26
28
22
25
20
21
17
19
7
12
5
6
3
4
11
1
52
57
48
51
46
47
13
15
41
44
14
16
9
8
10
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O

Fig. 4. Characterization of *E. coli* O157:H7 isolates using *eaeA* primers. (A) 1.5% agarose gel of amplified reaction mixtures. Lane M1 and M2: DNA markers; Lane 1 to 57: *E. coli* O157:H7 isolates; Lane 58, *E. coil* O157:H7 ATCC 43888; Lane 59: *E. coli* O157:H7 ATCC 43889; Lane 60: *E. coli* O157:H7 ATCC 43894, Lane 61: *E. coli* O157:H7 ATCC 43890. The arrow indicates the 354 bp *eaeA* amplification product. A total of 226 bands and 9 unique banding patterns was observed. (B) Dendogram of *E. coli* O157:H7 isolates and ATCC reference strains. Dendogram analysis yielded 25 genotypic patterns. The groupings are designated A to Y.

(A)


M1 1
30
10 kb
2 kb
1 kb
M2 31
62
10 kb
2 kb
1 kb

(B)

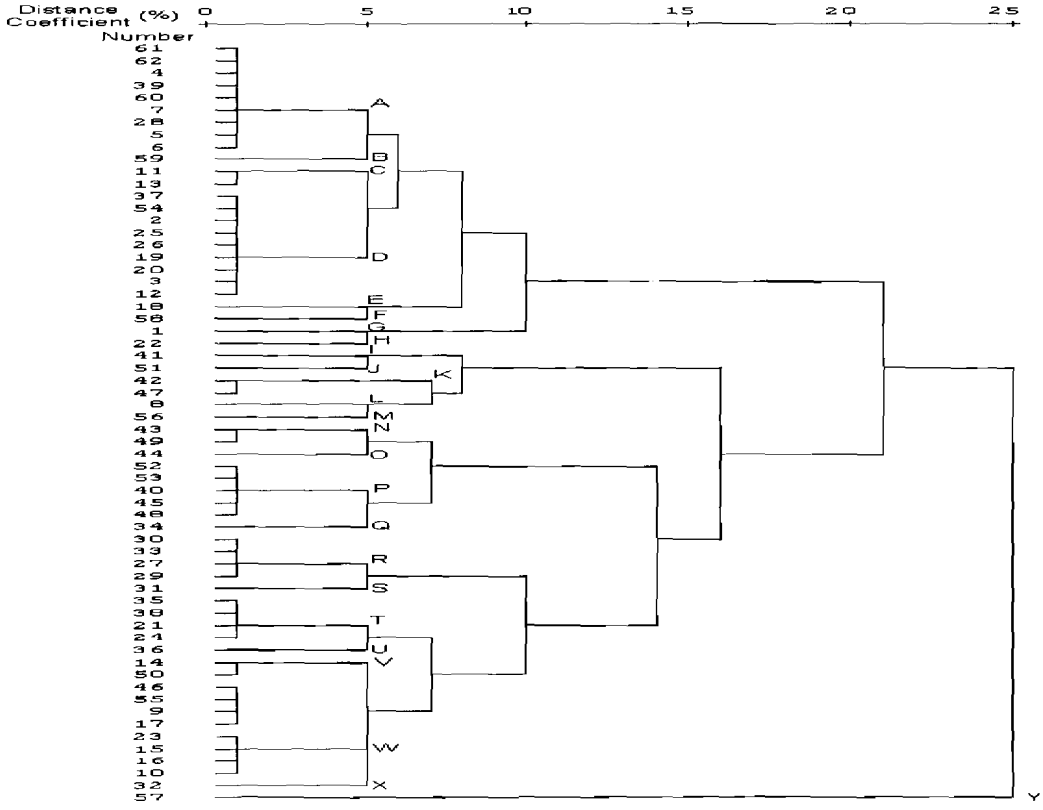

Distance Coefficient (%)
0
5
10
15
20
25
Number
61
62
4
39
60
7
28
5
6
59
11
13
37
54
2
25
26
19
20
3
12
18
58
1
22
41
51
42
47
8
56
43
49
44
52
53
40
45
48
34
30
33
27
29
31
35
38
21
24
36
14
50
46
55
9
17
23
15
16
10
32
57
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T
U
V
W
X
Y

Fig. 5. Characterization of *E. coli* O157:H7 isolates using ERIC-1 primers. (A) 1.5% agarose gel of amplified reaction mixtures. Lane M1 and M2: DNA markers; Lane 1 to 57: *E. coli* O157:H7 isolates; Lane 58: *E. coil* O157:H7 ATCC 43888; Lane 59: *E. coli* O157:H7 ATCC 43889; Lane 60: *E. coli* O157:H7 ATCC 43894, Lane 61: *E. coli* O157:H7 ATCC 43890. Agarose gel electrophoresis revealed a total the 153 bands and 17 unique banding patterns. (B) Dendogram of *E. coli* O157:H7 isolates and ATCC reference strains. Dendogram analysis yielded 22 genotypic patterns. The groupings are designated A to V.

(A)


M1 1
28
10 kbp
2 kb
1 kb
M2 29
55
10 kbp
2 kb
1 kb

(B)

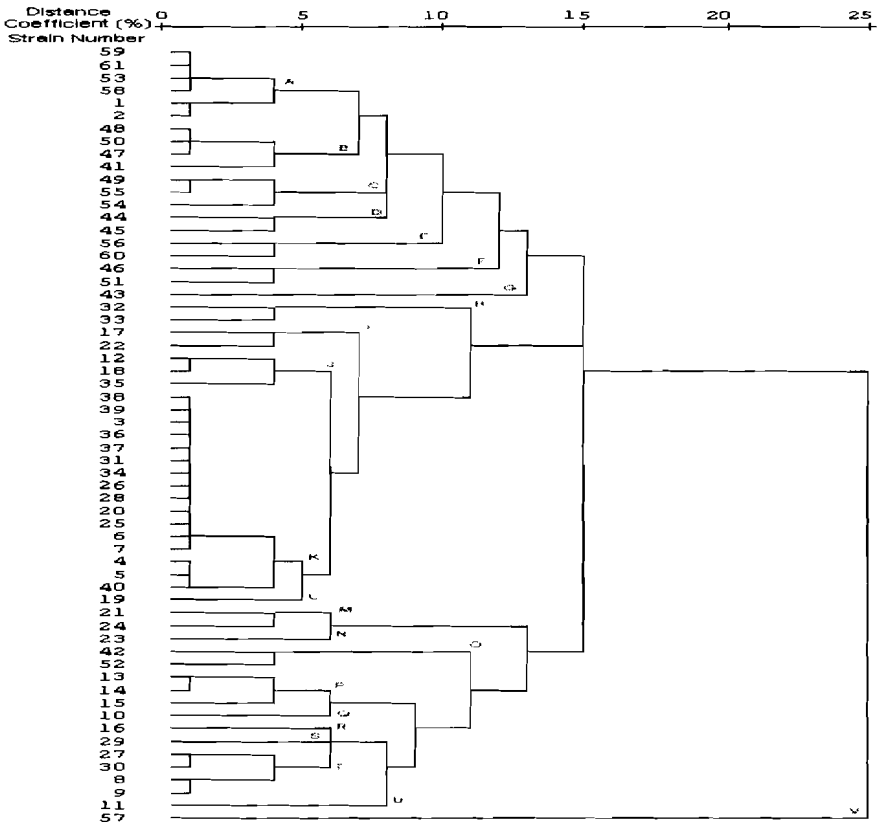
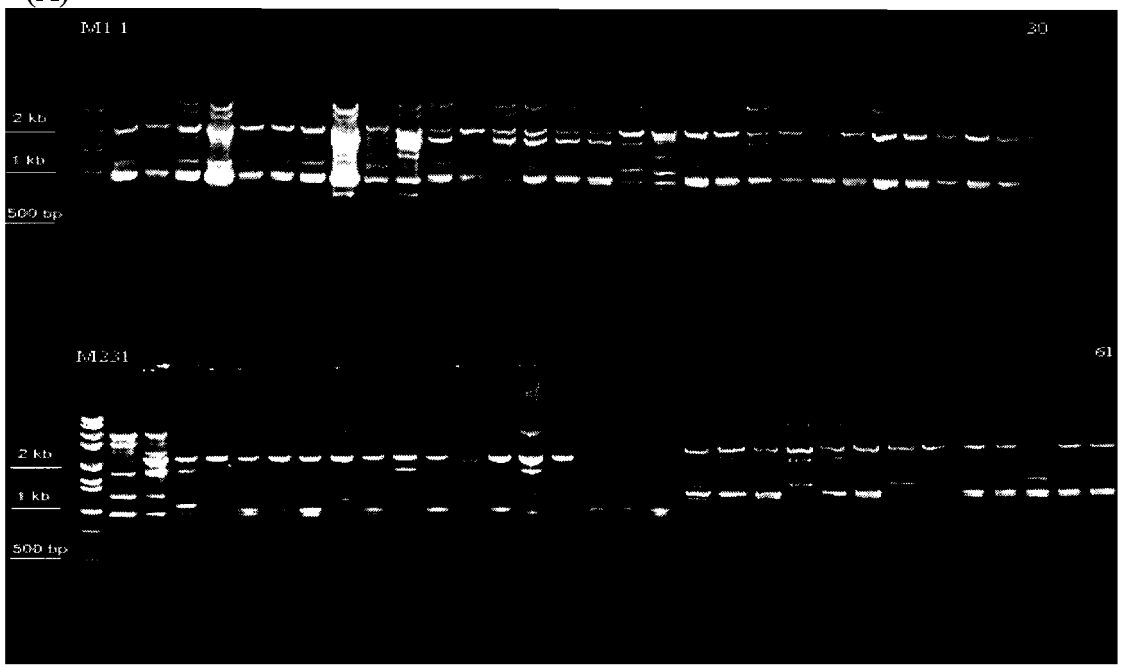

Distance
Coefficient (%)
Strain Number
0
5
10
15
20
25
59
61
53
58
1
2
48
50
47
41
49
55
54
44
45
56
60
46
51
43
32
33
17
22
12
18
35
38
39
3
36
37
31
34
26
28
20
25
6
7
4
5
40
19
21
24
23
42
52
13
14
15
10
16
29
27
30
8
9
11
57

Fig. 6. Characterization of *E. coli* O157:H7 isolates using 1254 primers. (A) 1.5% agarose gel of amplified reaction mixtures. Lane M1 and M2: DNA markers; Lane 1 to 57: *E. coli* O157:H7 isolates; Lane 58, *E. coil* O157:H7 ATCC 43888; Lane 59: *E. coli* O157:H7 ATCC 43889; Lane 60: *E. coli* O157:H7 ATCC 43894; Lane 61: *E. coli* O157:H7 ATCC 43890. A total of 394 bands and 27 unique banding patterns was determined by agarose gel electrophoresis. (B) Dendogram of the *E. coli* O157:H7 isolates and ATCC reference strains. Dendogram analysis yielded 46 genotypic patterns. The groupings are designated A to AT.

(A)


M1 1
30
2 kb
1 kb
500 bp
M231
61
2 kb
1 kb
500 bp

(B)

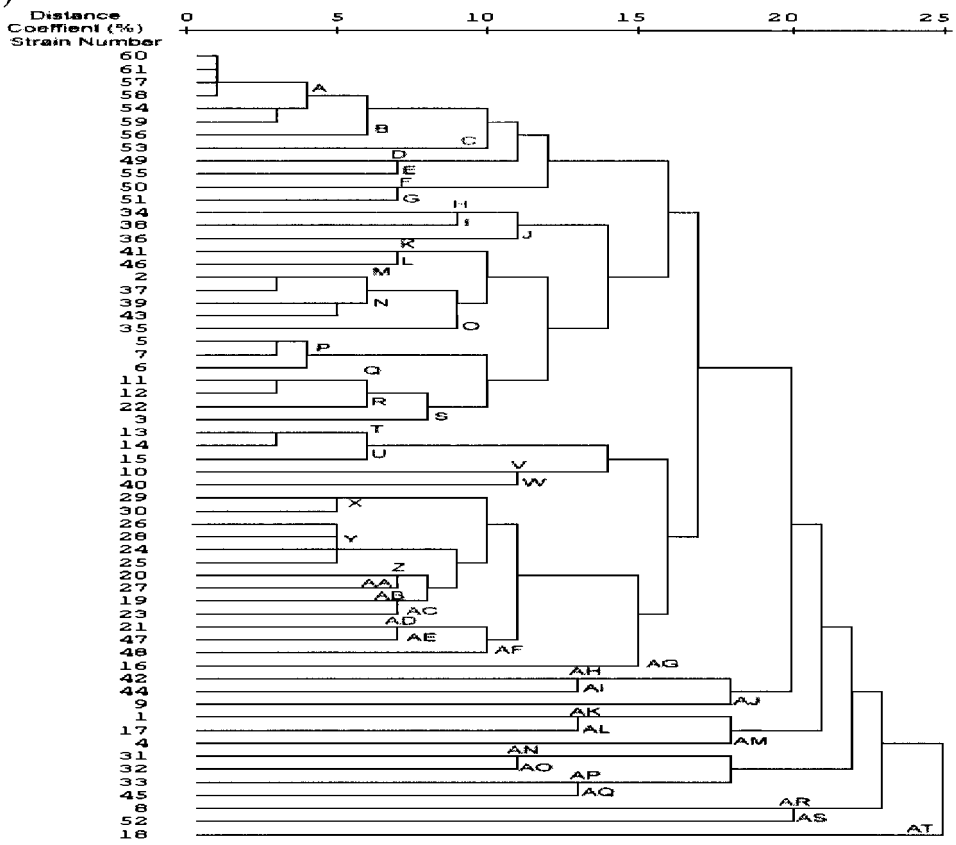
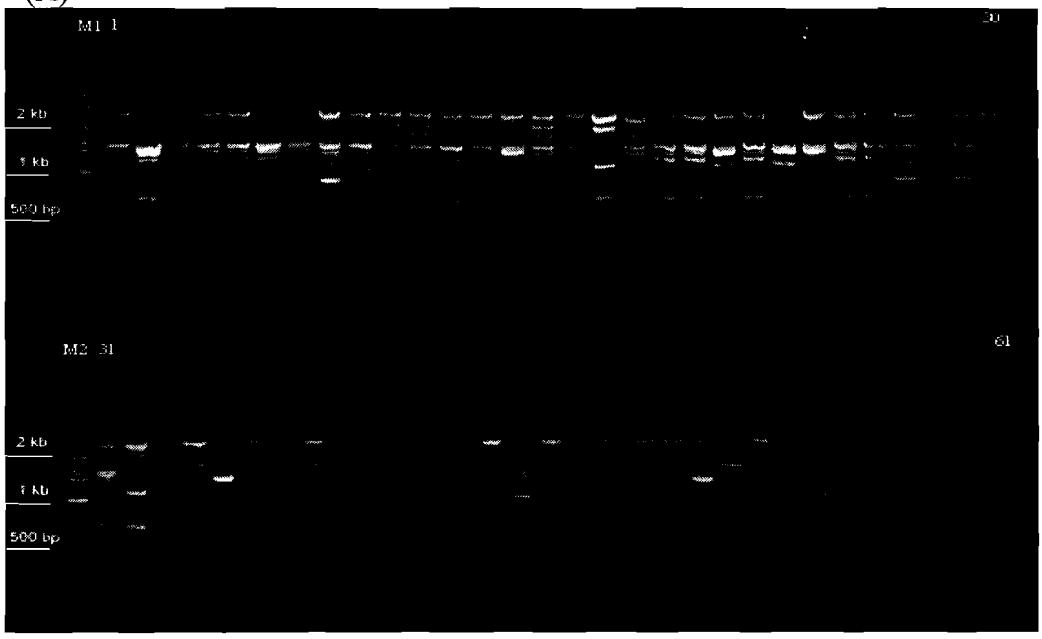

Distance
Coeffient (%)
Strain Number
0
5
10
15
20
25
60
61
57
58
54
59
56
53
49
55
50
51
34
38
36
41
46
2
37
39
43
35
5
7
6
11
12
22
3
13
14
15
10
40
29
30
26
28
24
25
20
27
19
23
21
47
48
16
42
44
9
1
17
4
31
32
33
45
8
52
18
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T
U
V
W
X
Y
Z
AA
AB
AC
AD
AE
AF
AG
AH
AI
AJ
AK
AL
AM
AN
AO
AP
AQ
AR
AS
AT

Fig. 7. Characterization of *E. coli* O157:H7 isolates using 1283 primers. (A) 1.5% agarose gel of amplified reaction mixtures. Lane M1 and M2: DNA markers; Lane 1 to 57: *E. coli* O157:H7 isolates; Lane 58, *E. coil* O157:H7 ATCC 43888; Lane 59: *E. coli* O157:H7 ATCC 43889; Lane 60: *E. coli* O157:H7 ATCC 43894; Lane 61: *E. coli* O157:H7 ATCC 43890. Agarose gel electrophoresis yielded 448 bands and 30 unique banding patterns. (B) Dendogram of the *E. coli* O157:H7 isolates and ATCC reference strains. Dendogram analysis yielded 46 genotypic patterns. The groupings are designated A to AT.

(A)


M1 1
30
2 kb
1 kb
500 bp
M2 31
61
2 kb
1 kb
500 bp

(B)

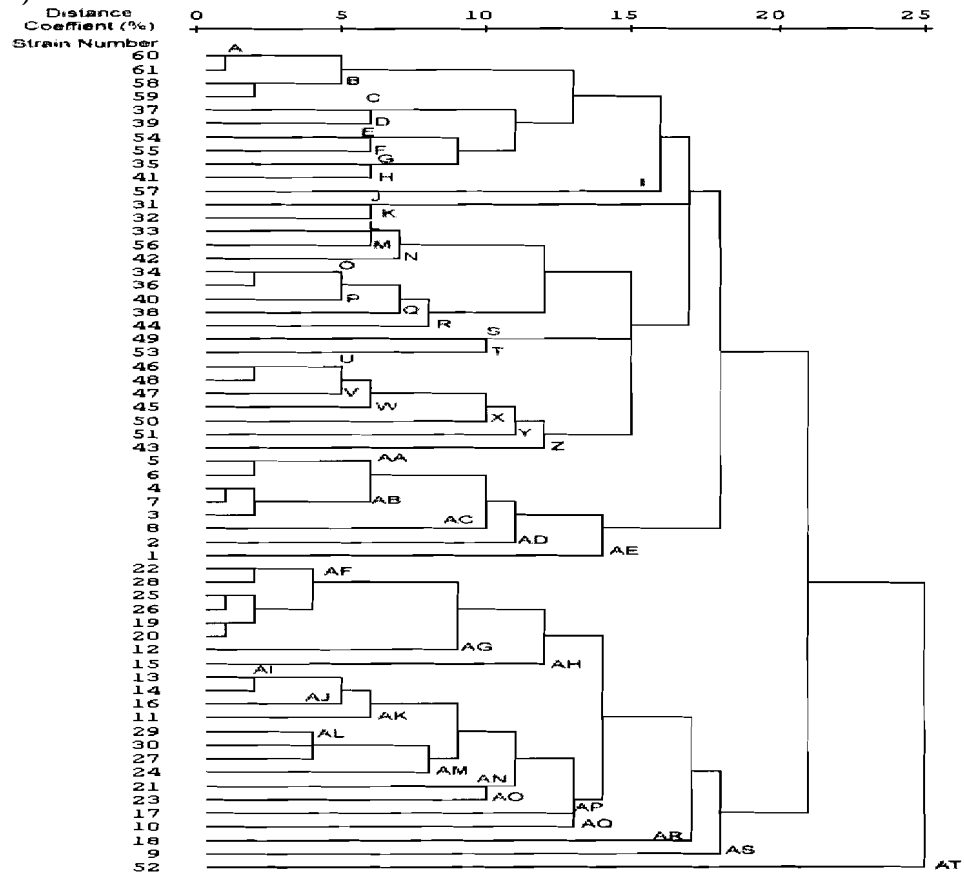
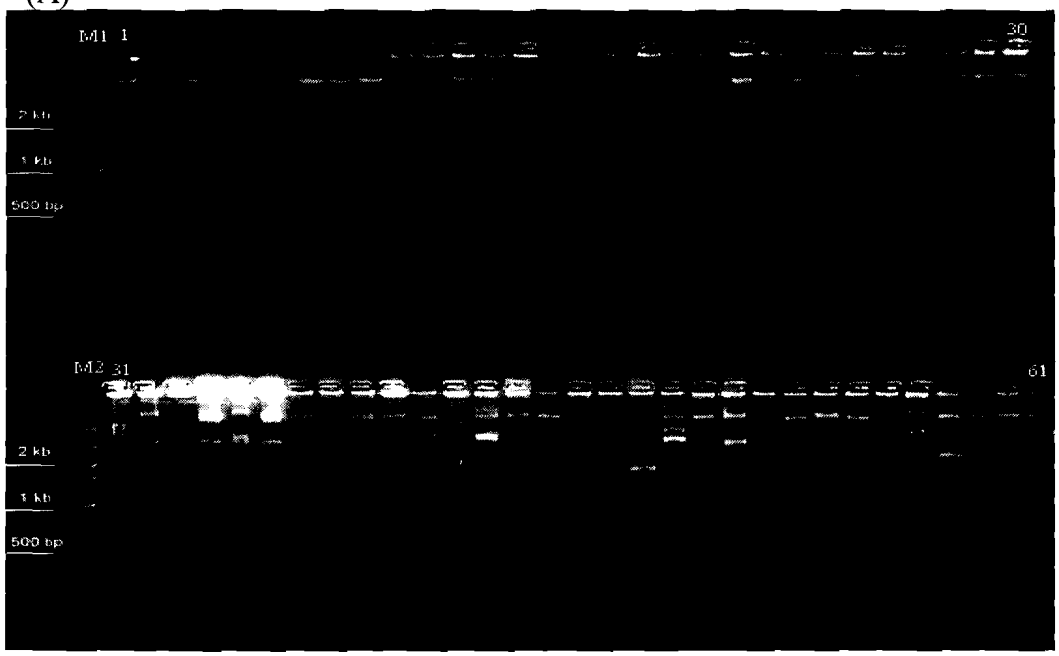

Distance
Coeffient (%)
Strain Number
0
5
10
15
20
25
60 A
61 B
58 C
59 D
37 E
39 F
54 G
55 H
35 I
41 J
57 K
31 L
32 M
33 N
56 O
42 P
34 Q
36 R
40 S
38 T
44 U
49 V
53 W
46 X
48 Y
47 Z
45 AA
50 AB
51 AC
43 AD
5 AE
6 AF
4 AG
7 AH
3 AI
8 AJ
2 AK
1 AL
22 AM
28 AN
25 AO
26 AP
19 AQ
20 AR
12 AS
15 AT
13
14
16
11
29
30
27
24
21
23
17
10
18
9
52

Fig. 8. Characterization of *E. coli* O157:H7 isolates using plasmid. (A) 0.7% agarose gel of plasmid profiles. Lane M1 and M2, DNA markers; Lane 1 to 57: *E. coli* O157:H7 isolates; Lane 58, *E. coil* O157:H7 ATCC 43888; Lane 59: *E. coli* O157:H7 ATCC 43889; Lane 60: *E. coli* O157:H7 ATCC 43894; Lane 61: *E. coli* O157:H7 ATCC 43890. A total of 153 bands and 10 unique banding patterns were observed. (B) Dendogram of *E. coli* O157:H7 isolates and ATCC reference strains. Dendogram analysis yielded 19 genotypic patterns. The groupings are designated A to S.

(A)


M1 1
30
2 kb
1 kb
500 bp
M2 31
61
2 kb
1 kb
500 bp

(B)

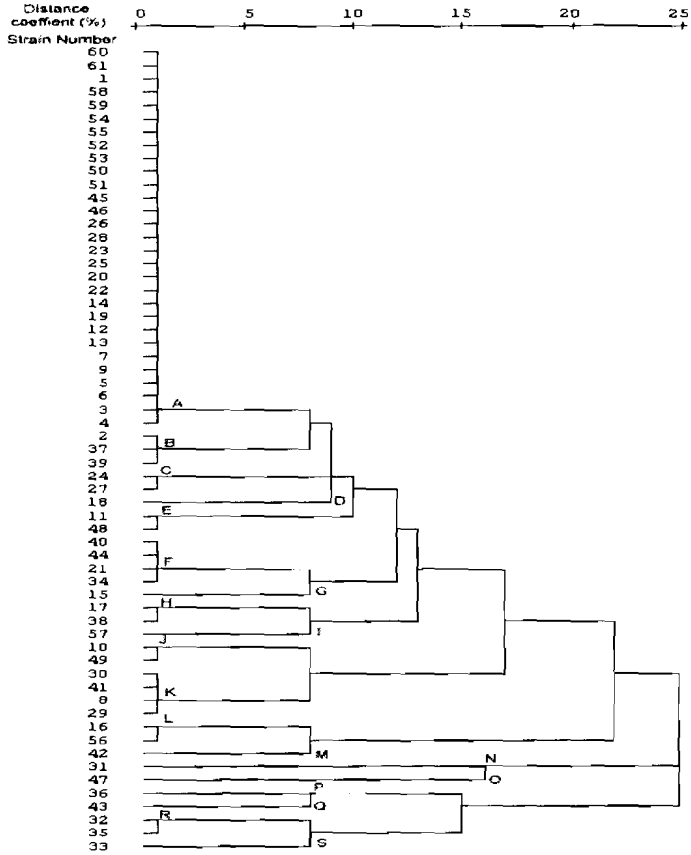

Distance coeffient (%)
0
5
10
15
20
25
Strain Number
60
61
1
58
59
54
55
52
53
50
51
45
46
26
28
23
25
20
22
14
19
12
13
7
9
5
6
3
4
2
37
39
24
27
18
11
48
40
44
21
34
15
17
38
57
10
49
30
41
8
29
16
56
42
31
47
36
43
32
35
33
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S

Table 4. Absorbance readings from DNA isolated from *E. coli* O157:H7 isolates and ATCC reference strains

Strain Number	260 nm	280 nm	Ratio	Concentration (mg/mL)
1	0.041	0.031	1.308	0.41
2	0.014	0.006	2.358	0.14
3	0.114	0.034	3.332	1.14
4	0.146	0.063	2.304	1.46
5	0.120	0.033	3.609	1.20
6	0.286	0.169	1.694	2.86
7	0.561	0.365	1.538	5.61
8	0.616	0.385	1.601	6.16
9	0.773	0.507	1.526	7.73
10	0.808	0.539	1.498	8.08
11	0.207	0.095	2.186	2.07
12	0.212	0.130	1.630	2.12
13	0.334	0.153	2.191	3.34
14	0.416	0.198	2.102	4.16
15	0.323	0.196	1.640	3.23
16	0.549	0.352	1.560	5.49
17	0.479	0.281	1.706	4.79
18	0.601	0.354	1.698	6.01
19	0.534	0.304	1.753	5.34
20	0.414	0.256	1.621	4.14
21	0.329	0.154	2.134	3.29
22	0.314	0.169	1.856	3.14
23	0.330	0.154	2.148	3.30
24	0.390	0.177	2.202	3.90
25	0.354	0.208	1.703	3.54
26	0.304	0.191	1.593	3.04
27	0.625	0.379	1.648	6.25
28	0.396	0.245	1.616	3.96
29	0.595	0.318	1.872	5.95
30	0.325	0.176	1.845	3.25
31	0.315	0.159	1.982	3.15
32	0.398	0.215	1.855	3.98
33	0.268	0.135	1.975	2.68
34	0.196	0.084	2.341	1.96
35	0.561	0.342	1.641	5.61

Table 4. Absorbance readings from DNA isolated from *E. coli* O157:H7 isolates and ATCC reference strains (Continued).

Strain Number	260 nm	280 nm	Ratio	Concentration (mg/mL)
36	0.864	0.575	1.503	8.64
37	0.424	0.241	1.76	4.24
38	0.531	0.308	1.725	5.31
39	0.454	0.274	1.658	4.54
40	0.720	0.455	1.583	7.20
41	0.484	0.246	1.965	4.84
42	0.595	0.317	1.878	5.95
43	0.302	0.140	2.158	3.02
44	0.217	0.093	2.332	2.17
45	0.759	0.404	1.566	7.59
46	0.663	0.421	1.574	6.63
47	0.894	0.611	1.462	8.94
48	0.678	0.381	1.779	6.78
49	0.332	0.196	1.646	3.32
50	0.833	0.516	1.616	8.33
51	0.372	0.191	1.950	3.72
52	0.178	0.102	1.749	1.78
53	0.284	0.126	2.264	2.84
54	0.185	0.077	2.410	1.85
55	0.564	0.345	1.633	5.64
56	0.601	0.405	1.483	6.01
57	0.047	0.009	5.177	0.47
ATCC 43888	0.727	0.388	1.871	7.27
ATCC 43896	0.923	0.665	1.389	9.23
ATCC 43889	0.799	0.535	1.493	7.99
ATCC 43894	1.086	0.784	1.385	10.9
ATCC 43890	0.598	0.345	1.733	5.98

Table 5. Summary of toxin genes amplification.

Strain Number	Toxin genes			
	stx1	*stx2*	*eaeA*	*hlyA*
1	+	+	+	+
2	-	+	+	+
3	-	+	+	+
4	+	+	+	+
5	+	+	+	+
6	+	+	+	+
7	+	+	+	+
8	+	-	-	+
9	-	-	-	-
10	+	-	-	-
11	+	+	+	+
12	+	+	+	+
13	-	+	-	-
14	-	+	-	-
15	-	-	-	-
16	+	+	-	-
17	+	+	+	+
18	-	+	-	-
19	-	+	+	+
20	+	+	+	+
21	+	+	-	+
22	+	+	+	+
23	+	+	-	-
24	-	+	-	-
25	-	+	+	+
26	+	+	+	+
27	-	+	-	-
28	-	+	+	+
29	-	-	-	-
30	+	-	-	-

Table 5. Summary of toxin genes amplification (Continued).

Strain Number	Toxin Genes			
	stx1	*stx2*	*eaeA*	*hlyA*
31	+	+	-	-
32	-	-	-	-
33	+	+	-	-
34	+	+	-	-
35	+	+	+	-
36	+	+	+	-
37	+	+	+	+
38	+	+	+	-
39	+	+	+	+
40	+	-	-	-
41	+	+	+	-
42	+	-	+	-
43	+	+	-	+
44	+	-	-	-
45	+	-	-	-
46	+	-	-	-
47	+	-	-	-
48	-	-	-	-
49	-	+	-	+
50	-	-	-	-
51	-	-	+	-
52	-	+	+	-
53	-	-	-	+
54	-	-	+	+
55	-	-	-	-
56	-	-	-	-
57	-	-	-	-

DISCUSSION

In epidemiological studies, it is important to trace the origin of outbreak strains and to keep a constant surveillance of potential outbreak strains. Epidemiological typing systems must be rapid and accurate if they are going to be useful in identifying organisms. Various typing methods have found utility in epidemiological studies, such as antimicrobial susceptibility testing, biotyping, serotyping, phagetyping, pulsed-field gel electrophoresis (PFGE), immunoblotting, ribotyping, plasmid profiling, and various PCR based approaches (15, 21, 36, 40, 46, 53).

E. coli O157:H7 has currently attracted worldwide attention because morbidity and mortality associated with this organism are rapidly increasing (5). Identification of this organism through PCR based approaches is generally accomplished by amplification of specific virulence genes (1, 26, 39, 45). The use of randomly amplified polymorphic DNA (RAPD) in genetic fingerprinting has increased in epidemiological studies in a variety of organisms due to its ability to yield fast results (11, 36, 38, 40, 53). However, due to lack of reproducibility, RAPDs have been the subject of much criticism. The purpose of this

research was to develop a modified RAPD approach which is able to identify specific virulence genes for identification purposes and provide genotypic information on *E. coli* O157:H7 in a single reaction. The results of this modified PCR based approach were also compared to conventional techniques used in epidemiological studies, such as plasmid profiling, ERIC-1 PCR, and conventional RAPDs.

Two arbitrary primers were used to determine genetic variation among the isolates in a conventional RAPD. The primers, designated 1254 and 1283, have been used in numerous studies to investigate genetic heterogeneity and distinguish serotypes within *E. coli* (38, 42, 57). Primer 1254 generated 46 unique groups whereas primer 1283 discriminated 46 groups. This approach, while useful in terms of examining genetic variation, makes no attempt to identify specific virulence genes. In development of the modified RAPD approach, it was first determined if specific virulence genes could be amplified. Table 5 summarizes the existence of the virulence genes tested. Overall, 13 isolates (22.8%) were positive for all of the virulence genes tested (*stx1*, *stx2*, *hlyA*, and *eaeA*). Decreasing the annealing temperature by 2°C for each reaction increased the

non-specific binding of the primers without losing the ability to specifically amplify the virulence gene in question. Thus, we were able to generate information on genetic variation and identify a specific virulence gene in a single reaction.

ERIC-1 PCR is the most widely used method in strain identification among the family *Enterobacteriaceae* and related organisms since it targets a specific repetitive element which exists only in the family *Enterobacteriaceae* (6, 51). Amplifying this element supplies strain specific information under high stringency conditions. In comparing ERIC-1 PCR to the modified PCR approach, genotypic information was gained from ERIC-1 PCR results but ERIC-1 PCR does not identify specific virulence genes.

E. coli O157:H7 strains have a large number of conjugative plasmids (34). However, they tend to lose their plasmids when there is no environmental pressure causing them to be maintained (34). Examining plasmid profiles of different strains yields information on genetic variation. Results gained from plasmid profiling of *E. coli* O157:H7 strains generated 17 groups. Thus, plasmid profiling was less discriminatory as compared to ERIC-1 PCR, RAPDs, and our modified method.

Comparing the discriminatory power of the presented methodologies, RAPDs were shown to have the highest resolution in demostrating genetic variation. However, RAPDs and ERIC-1 PCR were only able to yield information on the genetic relationship between each isolate, can could not amplify specific virulence genes. With the modified RAPD approach, the existence of a specific virulence gene and the genetic relationship among isolates were obtained in a single reaction.

The methodology presented herein may be useful for management of cattle feedyards by providing a method to investigate the geographic distribution of pathogenic strains of bacteria and serve as an identification tool. For future studies, a comparison of how plasmid profiles and genetic fingerprinting relate to antibiotic resistance would be interesting. A detailed investigation of phagetyping would also be useful to yield insight on the transmission of SLT genes and to acquire evolutionary patterns among the *Enterobacteriaceae* family.

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