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Characterization of an *Escherichia coli* O157:H7 Strain Producing Verotoxin 2 Isolated from a Patient in Korea

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Nine hundred patients diagnosed with diarrhea or hemorrhagic uremic syndrome in the Kyungpook Province, Korea, were examined from November 1998 to February 2000. One patient in Kumi appeared to possess the *Escherichia coli* O157:H7 strain, which is very important in clinical decision making and public health action. The isolated strain, an *E. coli* O157:H7 KM, contained a 60 MDa plasmid and typical virulence genes including the verotoxin 2 gene, *ehxA* gene (encoding enterohemorrhagic hemolysin), and *eae* (encoding attaching and effacing protein-intimin) gene. This strain produced only verotoxin 2. Pulsed field gel electrophoretic analysis showed that the genomic organization of the *E. coli* O157:H7 KM strain may differ greatly from those of representative strains previously reported in the United States and Japan.

Key words: Verotoxin, *eae*, *ehxA*, PFGE

Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 (22) was first recognized as a human pathogen in 1982 in the United States (32). Since then, at least thirty outbreaks of EHEC O157:H7 have been reported for the past ten years and people infected by this organism are estimated to be about 20,000 per year (5, 8, 18). In Japan, 9372 symptomatic patients were reported and 12 of them died in 1996 (5, 34). The most distinctive complication of EHEC is diarrhea-associated hemolytic uremic syndrom (HUS), a major cause of acute renal failure (15, 16). Other manifestations of EHEC infection can range from mild diarrhea to severe hemorrhagic colitis, thrombotic thrombocytopenic purpura, and death (16, 36). Ability to produce verotoxin and possess *eae* (*E. coli* attaching and effacing) and *ehxA* (*E. coli* enterohaemolysin) genes, and the presence of the 60 MDa plasmid (pO157) are important virulence-associated traits in this organism (4, 21, 30, 36).

Recently, EHEC O157:H7 infection has been reported frequently in many countries. Thus, it is regarded as a new emerging foodborne pathogen carefully monitored by the public health authorities (27). Because EHEC O157:H7 can be transmitted through contaminated food and water (34, 37), as well as by infected persons, clinical microbiologists are increasingly asked not only to identify, but also to type O157:H7 to clarify the chain of infection.

The typing methods currently employed include phage typing (2, 7, 14), multilocus enzyme electrophoretic typing (11), verotoxin genotyping (17, 28, 29), plasmid typing (29), random amplified polymorphic DNA fingerprinting (21, 26), and genomic DNA restriction fragment length polymorphism (RFLP) analyses (2, 9, 19).

To survey the infection rate of EHEC O157:H7 among diarrheal patients in Korea, we have carried out laboratory-based surveillance for the isolation of EHEC O157:H7 from November 1998 to February 2000. We isolated one strain of EHEC O157:H7 from 900 diarrheal patients. In this study, we investigated the presence of the verotoxin gene, *eae* gene, and *ehx*A gene by the polymerase chain reaction (PCR), of the 60 MDa plasmid, and the production of verotoxin. We also performed pulsed field gel electrophoresis (PFGE) to examine the genomic organization between this strain and other known EHEC O157:H7 strains (2, 19, 20, 31, 35).

Materials and Methods

Bacterial strains

A strain designated as *E. coli* O157:H7 KM was isolated from a 24 years old female patient diagnosed with diarrhea and HUS. *E. coli* O157:H7 ATCC43888 (9) and *E. coli* O157:H7 ATCC43895 strains (10) were from the Korea National Institute of Health and the American Type Culture Collection (VA, USA), respectively.

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Isolation of E. coli O157:H7 KM

An *E. coli* strain examined in this study was isolated from the patients at local hospitals in Kyungpook Province, from November 1998 to February 2000. The stool specimens of patients with diarrhea or HUS were inoculated onto MacConkey agar medium (Difco, Detroit, USA). Representative red or pink colonies were streaked onto MacConkey sorbitol agar medium, and transparent colonies were subject to standard biochemical test for the identification of *E. coli* (25). The conventional indole, methyl red, Voges-Proskauer, citrate, lysin decarboxylase test, and TSI reaction were performed. With API 20E test stripe and Vitek system (Biomerieux Vitek Inc., Hazelwood, USA), the identification of *E. coli* was confirmed. The strains identified as *E. coli* were screened for the absence of β-glucuronidase with Fluorocult *E. coli* O157:H7-Agar (Merck, Darmstadt, Germany). The β-glucuronidase negative strains were further screened for the presence of the O157 antigen by a slide agglutination test using *E. coli* O157 antiserum (KNIH, Korea). For O antigen serotyping, the test strain was grown in tryptic soy broth (Difco, Detroit, USA) at 37°C for 10 h without shaking. Cells were collected by centrifugation and resuspended in physiological saline. A part of this viable cell suspension was heated at 56°C for 30 min. The viable cell and the heated cell suspension were tested. For the serotyping of H antigen, the strain was passed three to four times through heart infusion (Difco, Detroit, U.S.A) based semisolid medium containing 0.3% agar to enhance its motility before H serotyping.

The strain was then grown in tryptic soy broth, and the cell was fixed by the addition of formaline (1% final concentration). The antisera for H serotyping (Denka Seiken Co., Tokyo, Japan) was used to determine the H serotype by a test tube agglutination test.

Transmission electron micrograph

The bacterial cells were prepared and observed under an electron microscope (JEM 100CX , JEOL, Japan) after negative staining.

Detection of verotoxin production

The strain was grown in CAYE medium (2% casamino acid, 0.6% yeast extract, 0.25% NaCl, 0.87% K_2HPO_4, 0.0005% $MgSO_4$, 0.0005% $FeCl_3$) with shaking at 37°C for 18 h. The culture was centrifuged, and the supernatant was tested for the presence of verotoxin 1 and verotoxin 2 by reversed passive latex agglutination (RPLA) test with a commercially available kit (Verotox-F SEIKEN, Denka Seiken Co., Japan). The experiment was performed according to the manufacturer's instruction.

Detection of verotoxin gene, ehxA gene and eae gene

The presence of the verotoxin gene, *ehxA* gene and *eae* gene in *E. coli* O157:H7 KM was tested by the PCR amplification method (12, 13, 33).

The template DNA was prepared by boiling of the culture broth (26, 33). PCR was carried out in a Progene thermal cycler (Techne, Princeton, UK) with a PCR amplification kit (TaKaRa, Otus, Japan). The primers for verotoxin genes were commercial products (TaKaRa, Otus, Japan). Primer EVT-1 and EVT-2 for verotoxin 1, EVS-1 and EVS-2 for verotoxin 2, and EVC-1 and EVC-2 for verotoxin 1 and 2 were used. The reaction was performed according to the manufacturer's instruction. For the amplification of the *ehxA* gene, PCR was performed with specific primers as described by Sandhu *et al.* (33) for 30 cycles under the following conditions: 96°C for 40 sec, 60°C for 1 min, and 70°C for 45 sec. For the amplification of the *eae* gene, PCR was performed with specific primers developed by Sandhu *et al.* (33) for 30 cycles under the following conditions: 94°C for 1 min, 59°C for 3 min, and 72°C for 5 min. Oligonucleotide primers were synthesized by Bioneer (Chungwon, Korea). The primers used are listed in Table 1. The PCR products were analyzed by 1.5% agarose gel electrophoresis and the molecular weight of the products was calculated using the TotalLab v1.00 program.

Isolation of plasmid

Plasmid DNA was extracted by the alkaline lysis method (3). The plasmid was electrophoresed on 0.7% agarose gel and visualized after ethidium bromide staining, and the apparent molecular weight of the plasmids was calculated against plasmid size markers in *E. coli* 327.

Pulsed field gel electrophoresis

Bacterial cells on an agar medium were directly embedded in low-melting-temperature agarose (Bio-Rad Lab., CA, USA). The DNA in each plug was digested with 30 U of *Xba* I (Roche Molecular Biochemical, Mannheim, Germany) at 37°C for 24 h. PFGE was performed on a 1% agarose gel in 0.5 ×TAE (Tris-Acetate-EDTA) buffer at 10°C, 200 V using a CHEF DRII apparatus (Bio-Rad lab.

Table 1. Primers used for the detection of the *eae* and *ehxA* genes

Gene	Primer	Sequence(5'-3')	Location within gene (bp)	PCR product size(bp)
eae	C-1	TCGTCACAGTTGCAGGCTGGT	803	1100
	C-2	CGAAGTCTTATCCGCCGTAAAGT	1912	
*ehx*A	CVD-I	AGCCGGAACAGTTCTCTCAG	381	526
	CVD-II	CCAGCATAACAGCCGATGTA	907	

CA, USA). For the separation of a whole genome, a linearly ramped switching time from 4 to 8 sec was applied for 11 h and then a linearly ramped switching time from 8 to 50 sec was applied for 9 h. The gel was photographed after ethidium bromide staining.

Results

Physiological and biochemical characteristics of E. coli O157:H7 KM

An *E. coli* O157:H7 strain was isolated among 900 patients with diarrhea or hemorrhagic uremic syndrome during the survey period by using biochemical and O:H serotyping, and designated as *E. coli* O157:H7 KM. This strain was bacilli type and had bipolar flagella (Fig. 1). The physiological and biochemical characteristics of *E. coli* O157:H7 KM are shown in Table 2. This strain showed the typical metabolic characteristics of the *E. coli* strain; indole (+), lactose (+), citrate (Simmon's) (-), and cellbiose (-). The results of sorbitol (-) and β-glucuronidase (-) correlate with the reactions of *E. coli* O157:H7 reported previously (25, 26).

According to the Aleksic's biotyping scheme (1), the *E. coli* O157:H7 KM belongs to biotype 5 [sorbitol (-), β-glucuronidase (-), Raffinose (-), Sucrose (-), Rhamnose (-), and Dulcitol (-)].

Detection of verotoxin genes and verotoxin

The presence of verotoxin genes in *E. coli* O157:H7 KM was examined by PCR. The results of PCR assay for the verotoxin genes are shown in Fig 2. No PCR product for the verotoxin 1 gene was observed (lane 3). PCR product of 404 bp was amplified for the verotoxin 2 gene (lane 2) and 176 bp was amplified for the common region of verotoxin 1 and verotoxin 2 genes (lane 1). The PCR product was cloned in the pT7Blue T-Vector and sequenced. The nucleotide sequence of the PCR product for the verotoxin gene of *E. coli* O157:H7 KM showed 99% identity with that of the Shiga like toxin type II (Verotoxin 2) of *E. coli* bacteriophage 933W (10)(data not shown). *E. coli* O157:H7 KM strain had the ability to produce verotoxin 2 only (Fig. 3). It appears that *E. coli* O157:H7 KM only has verotoxin 2 gene.

Detection of the eae and ehxA genes

The *eae* gene, known to be located on the EHEC chromosome, encodes the 94-97 KDa outermembrane protein intimin, an intestinal adherence factor which plays a role in intestinal colonization (9, 23, 24).

The *ehxA* gene located on the EHEC pO157 plasmid (6, 34), encode hemolysin, which lyze erythrocytes, and it can serve as a source of iron, which enhances the growth of *E. coli* O157:H7. Fig. 4 shows that the products amplified by PCR using the primer C-1 and C-2 for the *eae*

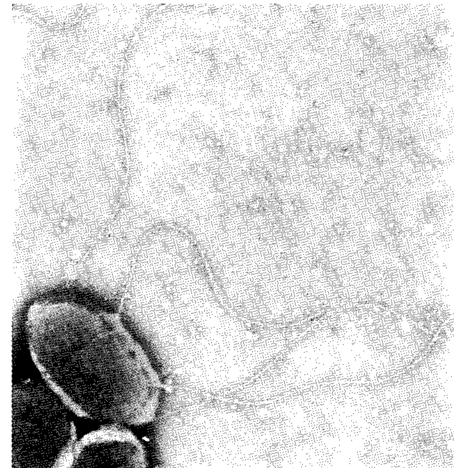

Fig. 1. Transmission electron micrograph of *E. coli* O157:H7 KM

Table 2. Physiological and biochemical characteristics of *E. coli* O157:H7 KM[a]

Factor	*E. coli* O157:H7 KM
2,4,4-Trichloro-2-Hydroxydiphenylether(DP3)	-
Oxidation/Fermentation(Glucose)	+
Acetamide	-
Esculin	-
Indoxyl-β-D-glucoside(PLI)	-
Urea	-
Citrate	-
Malonate	-
Tryptophan	-
Polymyxin B peptone	-
Lactose	+
Maltose	+
Mannitol	+
Xylose	+
Sorbitol	-
Raffinose	-
Sucrose	-
Rhamnose	-
Dulcitol	-
Inositol	-
Adonitol	-
p-Coumaric	+
H_2S(Hydrogen thiosulfate)	-
O-Nitrophenyl-β-D-Thio-Isopropyl-Galactopyranoside	+
L-Arabinose	+
Glucose	+
Arginine	-
Lysine Decarboxylase	+
Ornithine	+
β-glucuronidase	-

[a]Results were obtained after 24 h at 37°C but those of raffinose, sucrose, rhamnose and dulcitol were after 7 days.

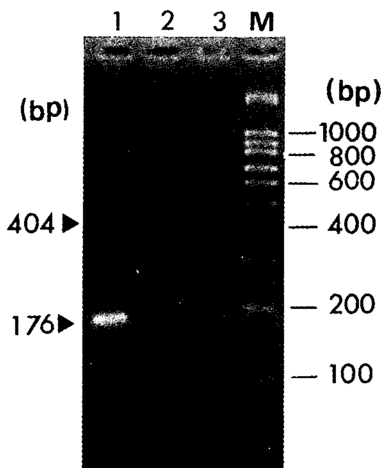

Fig. 2. PCR amplification for the detection of verotoxin genes in *E. coli* O157:H7 KM. Lanes: 1, verotoxin 1 and 2 common gene; 2, verotoxin 2 specific gene; 3, verotoxin 1 specific gene; M, 100 bp ladder (JBI, Taegu, Korea). Arrowheads indicate the specific products of verotoxin 2. Molecular sizes are indicated on the right in base pairs.

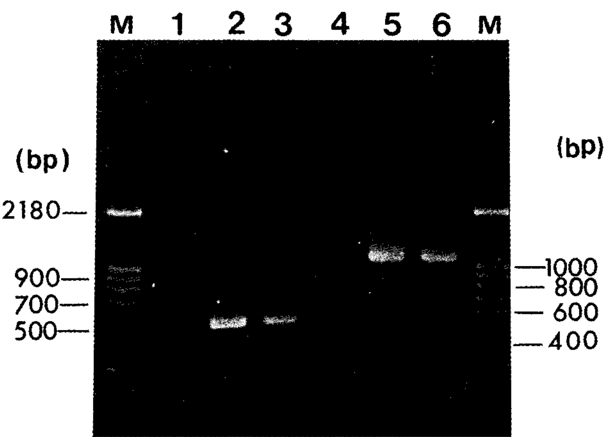

Fig. 4. PCR amplification for the detection of the *eae* and *ehxA* genes from *E. coli* O157:H7 KM. Lanes: 1, negative control; 2, *ehxA* positive strain (*E. coli* O157:H7 ATCC43895); 3, *E. coli* O157:H7 KM; 4, negative control; 5, *eae* positive strain (*E. coli* O157:H7 ATCC43888); 6, *E. coli* O157:H7 KM; M, 100 bp ladder (JBI, Taegu, Korea). Molecular sizes are indicated on the left and right (the same as those on the left) in base pairs.

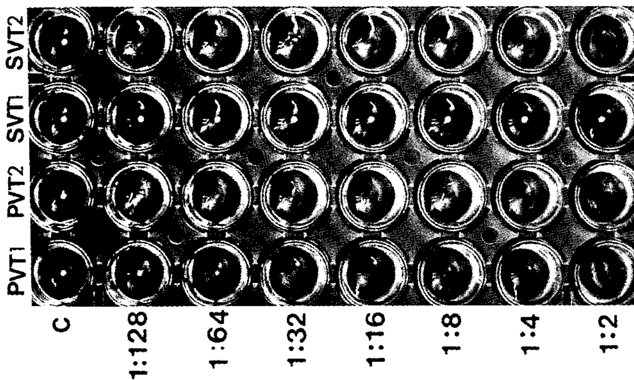

Fig. 3. Typing of verotoxin produced by *E. coli* O157:H7 KM by RPLA. Two positive controls for verotoxin 1 and 2 (Pvt 1, Pvt 2) and adequately prepared sample were serially diluted 1:128. At row Pvt 1, the agglutination was observed by the dilution rate of 1:32 and at row Pvt2, by 1:64. At the diluted sample of the *E. coli* O157:H7 KM, the agglutination for verotoxin 2 (Svt 2) occurred for the 1:128 diluted sample but not for verotoxin 1 (Svt 1).

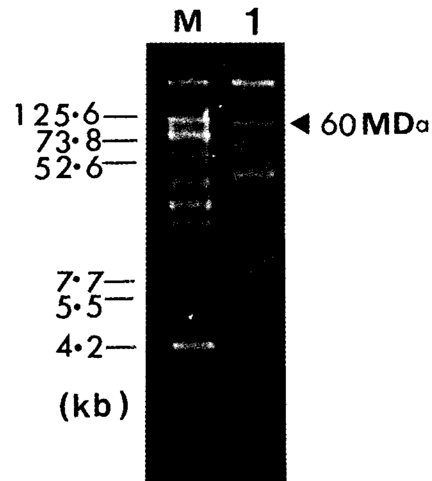

Fig. 5. Plasmid profile of *E. coli* O157:H7 KM. Lanes: 1, plasmid DNA of *E. coli* 327 (molecular weight size marker); 2, *E. coli* O157:H7 KM. The 60 MDa plasmid was indicated by the arrowhead. Molecular sizes are indicated on the left in kb.

gene and CVD-1 and CVD-2 for the *ehxA* gene in *E. coli* O157:H7 KM (Table 1.). PCR products show 526 bp for the *ehxA* gene (lane 3) and 1,100 bp for the *eae* gene (lane 6) as expected.

Detection of 60 MDa plasmid of E. coli O157:H7 KM

All *E. coli* O157:H7 strains have been known to contain a highly conserved 60 MDa plasmid (4, 6, 30). It contains genes encoding several proteins implicated in EHEC pathogenecity such as an EHEC hemolysin (*ehxA*), catalase-peroxides (*katP*), serine protease (*espP*), and type II secretion system. Fig. 5 shows that the *E. coli* O157:H7 KM strain has the 60 MDa plasmid with other plasmids.

PFGE patterns of E. coli O157:H7 KM

PFGE was applied for molecular typing of the isolated *E. coli* O157:H7 KM strain. By restriction endonuclease *Xba*I digestion analysis, the genome of *E. coli* O157:H7 KM showed about 20 different fragments ranging from 20 to 436.5 kb. The fragments, larger than 436.5 kb in size, were not produced from *E. coli* O157:H7 KM. The PFGE patterns of *E. coli* O157:H7 KM were different from those of *E. coli* O157:H7 ATCC43888 of the United States in more than seven bands (Fig. 6, lanes 1, 2).

All of the different bands were larger than 100 kb in size. The sizes of the different bands were one band of

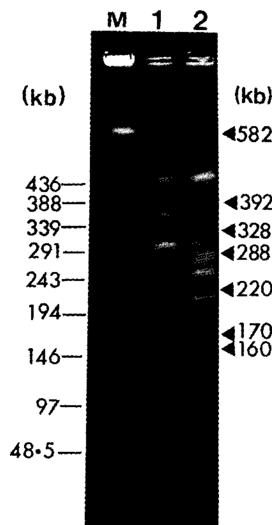

Fig. 6. The *Xba* I PFGE patterns of *E. coli* O157:H7 KM. Lanes: 1, *E. coli* O157:H7 ATCC 43888; 2, *E. coli* O157:H7 KM; M, DNA Size Standard - Lambda (Bio-Rad Laboratories, Hercules, CA). The sizes of different bands between two strains are indicated by arrowheads. Molecular sizes are indicated on the left in kb.

582 kb , 220 kb, and 170 kb in lane 1, and 392 kb, 328 kb, 288 kb, and 160 kb in lane 2.

Discussion

In this study, we first report the isolation and characterization of *E. coli* O157:H7 strain producing only veroxin 2 in Korea (21). The biochemical characteristics of *E. coli* O157:H7 KM showed those of typical *E. coli,* and the strain appeared to belong to biotype 5 as classified by Aleksic (1). This biotype is the type of isolate which appears most frequently in Japan and the United States (1, 5). For the full pathogenicity of *E. coli* O157:H7, the ability to produce one or both verotoxins (verotoxin 1 and verotoxin 2), the presence of the *eae* and *ehxA* gene, and the possession of the 60 MDa plasmid are very important (27). Typically, most strains of *E. coli* O157:H7 produce verotoxin 2 alone, but some produce verotoxin 1 or both verotoxin 1 and verotoxin 2. Verotoxin 2 is more important than verotoxin 1 in the progression of *E. coli* O157:H7 infection to HUS (27). The nucleotide sequence of the verotoxin 2 gene of *E. coli* O157:H7 KM suggests that it might be derived from *E. coli* bacteriophage 933W(10). *E. coli* O157:H7 KM possessed the *eae* gene, *ehxA* gene, and 60 MDa plasmid. Based on these results, *E. coli* O157:H7 KM may be a typical *E. coli* pathogenic strain.

PFGE has become a standard technique for typing EHEC O157:H7 (2). Compared to the *Xba* I PFGE patterns of other *E. coli* O157:H7 isolates previously reported, those of the *E. coli* O157:H7 KM showed a unique pattern.

We compared, though not directly, the *Xba* I PFGE patterns of *E. coli* O157:H7 KM with those of *E. coli* O157:H7 reported by Izumiya *et al.* (19) and Barrett *et al.* (2). The PFGE patterns of *E. coli* O157:H7 KM were similar to those of *E. coli* O157:H7 isolated from an outbreak in Sakai city, Osaka Prefecture, Japan, with a size below 436.5 kb except for 170 kb as a whole. However, they were differed in sizes greater than 436.5 kb. The size of the different bands in patterns of Sakai isolates were 582 kb, 485 kb, and 170 kb. The PFGE patterns of *E. coli* O157:H7 isolated from other countries were reported to be larger than 436.5 kb in size (17, 19, 20, 23, 24). It appears that the *E. coli* O157:H7 KM isolate is a novel type strain.

During the 16 month investigation period, we detected only one strain of *E. coli* O157:H7 among 900 patients. These results suggest that infection and colonization with this organism might occur at a very low rate in Korea. Recently, the frequency of infection by *E. coli* O157:H7 is continuously increasing worldwide (8, 22). In Korea, *E. coli* O157:H7 can already be considered a major pathogen involved in food poisoning and a harmful health threat in the near future. Extensive epidemiological and bacteriological investigation for hemorrhagic colitis, HUS, and uncomplicated diarrhea is required in Korea to determine their specific cause.

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