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Isolation and characterization of *Escherichia coli* O157 from retail beef and bovine feces in Thailand

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Abstract

Antibody to *Escherichia coli* O157 lipopolysaccharide was detected in the sera of healthy individuals more frequently in Southern Thailand than in Japan. The result suggested possible exposure of Thai people to *E. coli* O157. *E. coli* O157:H7 or O157:H$^-$ was isolated from four of 95 retail beef and one of 55 bovine feces samples collected in Southern Thailand by enrichment culture followed by immunomagnetic bead separation. Four of the five strains carried the stx_2 gene alone or in combination with the stx_1 gene. The strains were shown to be genetically distinct by an arbitrarily primed PCR method.

Keywords: Shiga toxin; Beef; Enterohemorrhagic *Escherichia coli*

1. Introduction

Escherichia coli O157 strains producing flagella of H7 serotype (O157:H7) or not producing flagella (O157:H$^-$), hereinafter abbreviated as O157:H7/H$^-$, are important foodborne pathogens. The organisms usually produce one or both of two Shiga toxins (Stxs: Stx1 and Stx2) and can cause not only diarrhea but more serious diseases such as hemorrhagic colitis and hemolytic uremic syndrome [1]. Natural reservoirs of these organisms are cattle and other domestic animals [2]. Therefore, beef, dairy products, and related foods are the primary source of *E. coli* O157:H7/H$^-$ infection in humans [2]. Isolation of *E. coli* O157:H7/H$^-$ has been reported very often from developed countries [1,2]. A big outbreak and many sporadic cases due to *E. coli* O157:H7 occurred in 1996 in Japan [3]. However, isolation of *E. coli* belonging to serogroup O157 has rarely been reported in Asian countries except Japan; isolation of *E. coli* O157 from clinical sources in India, China, Korea, and Hong Kong has been briefly reported [1,4–6]. *E. coli* of O157 serogroup was not detected in a survey for Stx-producing *E. coli* in retail meat and farm animals in Thailand, which was reported in 1990 [7]. Recently, beef marketed in Malaysia was shown to be contaminated by *E. coli* O157:H7 [8]. We therefore postulated that this organism may be distributed in Thailand. The level of serum antibody to O157 lipopolysaccharide may help in assessing the level of exposure to *E. coli* O157 [9]. In this study, we first found that the proportion of the healthy individuals with the antibody against *E. coli* O157 is higher in Southern Thailand than those in Japan. This encouraged us to examine retail meat and bovine feces in Southern Thailand for the presence of *E. coli* O157:H7/H$^-$ by a sensitive method utilizing immunomagnetic beads coated with anti-O157 antibody.

2. Materials and methods

2.1. Determination of anti-O157 antibody titer

A direct agglutination test was performed to determine anti-O157 antibody titer in the test serum. The test serum was immobilized by heating at 56°C for 30 min. The im-

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mobilized serum was first diluted 1:10 and then two-fold dilutions were prepared with sterile saline in U-bottom microtiter plates and were made to react with an equal amount (0.025 ml) of the antigen solution at 50°C overnight. *E. coli* O157:H7 strain 96-1 capable of producing Stx1 and Stx2, which was isolated from a patient with bloody diarrhea in Kyoto Prefecture in 1996, was used to prepare the antigen. The strain was grown on brain heart infusion agar (Difco Laboratories, Detroit, MI, USA) at 37°C for 18 h. The cells were harvested and suspended in formol-saline (0.85% NaCl solution containing 1% formalin) and heated at 100°C for 2 h. The heat-killed cells were washed three times with sterile saline (0.85% NaCl solution). The washed cells were suspended in formol-saline and the cell concentration was adjusted so that the turbidity corresponded to McFarland nephelometer tube number 4. Agglutinating titer was recorded as the reciprocal of the highest dilution of the test serum that gave any evidence of agglutination.

2.2. Isolation of E. coli O157

Beef was purchased from fresh markets, supermarkets, or a department store and bovine feces were collected from the cattle during the period between May and October 1998 in Songkla Province, Thailand. Fresh bovine feces excreted from young to adult animals (dairy and beef cattle) were collected into sterile plastic containers, transferred immediately to the laboratory at room temperature, and processed within 2 h after collection. Twenty-five gram of beef or fecal specimen was homogenized in a stomacher with 225 ml of EC broth (Difco Laboratories) containing novobiocin (20 mg ml^{-1}) for 2 min and incubated at 42°C for 18 to 24 h. Then 1 ml of the culture was treated with immunomagnetic beads coated with anti-O157 antibody (Dynabeads® anti-*E. coli* O157, Daynal A.S., Oslo, Norway) for 10 min with continuous agitation according to the manufacturer's specification and inoculated onto sorbitol MacConkey agar (Oxoid, Ltd., Basingstoke, England). After 18- to 24-h incubation at 37°C, up to five colorless colonies were transferred onto CHROMagar® O157 (CHROMagar Microbiology, Paris, France) and incubated at 37°C overnight. The purple colonies were examined by the standard biochemical tests for confirmation of *E. coli* [10]. Those identified as *E. coli* were subjected to the slide agglutination test using heat-killed preparation of the cells and anti-O157 serum (*E. coli* O antiserum O157, Denka Seiken, Co., Ltd., Tokyo, Japan) as reported previously [8]. Presence of the *rfbE* gene in the test strains was examined by the PCR method as described previously [11].

2.3. Characterization of E. coli O157

The motility of the five strains of *E. coli* O157 was enhanced by passing the strains three to four times through heart infusion (Difco Laboratories)-based semi-solid medium containing 0.5% agar before their H serotype was determined using a commercially available kit as described previously [8]. Possession of the *stx* gene was examined by the PCR method and production of Stx was examined by a reversed passive latex agglutination method using Verotox-F Seiken kit (Denka Seiken) as described previously [8]. Presence of the *eae* gene and the DNA sequence specific to the 60-MDa plasmid were examined by the PCR methods as described earlier [5]. Plasmid profiles of the test strains were examined by alkaline plasmid extraction method [12] followed by 0.7% agarose gel electrophoresis.

2.4. Genetic fingerprinting

For ribotyping, genomic DNA of the test strain was purified as described previously [8]. Purified DNA was digested with *Hin*dIII, *Mlu*I or *Nco*I. The digested DNA was electrophoresed in 1% agarose gel and blotted onto a nylon membrane. The rRNA gene probe was synthesized by a PCR amplification using EDL933 genomic DNA as the template and primers 5′-AGAGTTTCCTGGCTCAG-3′ (position 8–27 of *E. coli* 16S rRNA) and 5′-GGTTACCTTGTTACGACTT-3′ (position 1510–1493) as described [13]. The probe was labeled with digoxigenin using DIG DNA Labeling Kit (Boehringer Mannheim, Germany). Hybridization was performed at 42°C and the probe-positive bands were detected colorimetrically with the DIG Nucleic Acid Detection Kit (Boehringer Mannheim) according to the manufacturer's specification.

Arbitrarily primed PCR (AP-PCR) was performed using RAPD analysis primer set (Pharmacia Biotech, Inc., Uppsala, Sweden) as described previously [14].

3. Results and discussion

3.1. Serum titers against E. coli O157 for healthy individuals

Human sera were obtained from healthy individuals in Hat-Yai, Songkla Province, Thailand in February 1998 and in Kyoto Prefecture, Japan between September and December 1996. There were 14 sporadic cases of *E. coli* O157:H7 infection in Kyoto Prefecture in 1996 whereas no case due to *E. coli* O157 has been reported in Thailand. We employed a direct agglutination test to determine anti-O157 antibody titers. Little information is available as to the agglutinating titer to *E. coli* O157 for the sera from healthy individuals. Notenboom et al. [15] reported that the average agglutinating titer to *E. coli* O157 for *E. coli* O157 culture-positive patients was 660 and the titers of negative controls, except for those experienced infection by *Brucella abortus* carrying a cross-reacting antigen, were less than eight. We obtained the convalescent serum

Table 1
Detection of anti-O157 antibody in the serum samples of healthy individuals in Kyoto Prefecture, Japan and Songkla Province, Thailand

Age group	No. of samples			
	Japanese		Thai	
	Examined	Positive	Examined	Positive
<1	27	0 (0)[a]	0	n.a.[b]
1–6	68	0 (0)	0	n.a.
7–12	28	0 (0)	0	n.a.
13–19	19	0 (0)	18	5 (28)
20–59	75	5 (6.7)	87	17 (19.5)
60–79	39	1 (2.6)	0	n.a.
Total	256	6 (2.3)	105	22 (21.0)

[a]Percentage of positive samples.
[b]n.a., not applicable.

of an *E. coli* O157 culture-positive patient in Kyoto prefecture in 1996. The titer of this serum was 160. We therefore recorded the result by arbitrarily defining that sera with the titer of 10 or more were positive for anti-O157 antibody (Table 1). Age-matched comparison was possible for those at ages between 13 and 59. The percentage of positive samples was much higher for Thai samples than for Japanese samples. The anti-O157 titers for the positive serum samples were 10 (five samples) and 40 (one sample) for the Japanese and 10 (18 samples), 20 (three samples), and 40 (one sample) for the Thai. When the cutoff titer for the positive sample was increased to 20 or 40, the percentage of positive samples was still higher for Thai samples or equal for Thai and Japanese samples, respectively. Although non-*E. coli* organisms that carry cross-reacting O antigens may have contributed in part to the positive titer [1,15], our results may be interpreted to suggest that Thai people may have been exposed to *E. coli* O157 more often than or as often as were the Japanese people during *E. coli* outbreaks in 1996. The result prompted us to examine the possibility that *E. coli* of O157 serogroup may be distributed in Southern Thailand.

3.2. Isolation of E. coli O157

Ninety-five beef samples and 55 bovine feces samples were examined by a series of isolation steps including the separation with immunomagnetic beads coated with anti-O157 antibody. *E. coli* strains of serogroup O157 were isolated from four beef samples (strains T1, T2, T4, and T12) and one bovine feces sample (strain T13). These five strains carried the *rfbE* gene essential for O157 antigen expression.

3.3. Characterization and comparison of the isolated O157 strains

Two strains were judged nonmotile and thus recorded as H^- and three strains were typed to H7 (Table 2). The five strains of *E. coli* O157:H7/H^- were also examined for virulence-associated characteristics. These results are summarized in Table 2. All five strains carried the *eae* and 60 MDa plasmid sequences but genetic potential and actual ability to produce Stx varied among the strains. Three randomly selected colonies of each of the test strains were examined for the *stx* gene and Stx. The results obtained with three colonies were identical. Two strains carried both stx_1 and stx_2 genes and two strains had the stx_2 gene. However, the production of Stx2 from three stx_2-positive strains could not be confirmed. There are a number of possible explanations for this. The *stx* genes in some strains are unstable [16]. The *stx* gene in the three strains could have been lost during the growth for Stx assay. A stx_2-positive O157:H7 strain that does not produce Stx2 at the level detectable by an immunological method has been reported [6]. However, the Verotox-F assay, which was employed to determine Stx production in this study, is very specific and sensitive [17]. Therefore, the above three strains may produce no or very little Stx2. Alternatively, the stx_2 genes of the three strains may encode variant Stx(s) not detectable by the Verotox-F assay. We are currently investigating these possibilities. Strain T4 carried neither the stx_1 nor stx_2 gene. O157:H7/H^- strains that lack the *stx* gene are known to exist [18,19].

Plasmid profiles of the five Thai strains were compared (Fig. 1). Strain EDL933, an O157:H7 strain isolated from an outbreak in Michigan in 1982 and known to carry a typical 60 MDa plasmid [20,21] was employed as the control strain (Fig. 1, lane 7). Thai strains other than T13 carried plasmids similar in mobility to the 60 MDa plasmid of EDL933. Strain T2 (Fig. 1, lane 3) carried an additional, large plasmid. Strain T13 (Fig. 1, lane 6) contained two plasmids exhibiting different mobility from that of the 60 MDa plasmid of EDL933.

Table 2
Characteristics of five strains of *E. coli* O157 isolated from beef and bovine feces in Thailand

Strain	H serotype	Detection of[a]:					
		stx_1 gene	Stx1	stx_2 gene	Stx2	*eae* gene	60 MDa plasmid sequence
T1	H^-	+	+	+	+	+	+
T2	H7	−	−	+	−	+	+
T4	H7	−	−	−	−	+	+
T12	H7	−	−	+	−	+	+
T13	H^-	+	+	+	−	+	+

[a]+, detected; −, not detected.

The five Thai strains were also compared by genetic fingerprinting methods. Various *E. coli* strains have been compared by ribotyping methods. Although digestion of genomic DNA by *Hin*dIII has been used very often for *E. coli* ribotyping, *Nco*I or *Mlu*I digestion may serve better to distinguish O157 strains [22,23]. All five strains exhibited identical fingerprinting patterns for all these restriction enzymes examined. These patterns were identical with those of EDL933 (data not shown). Therefore, the test strains were compared next by an AP-PCR method. Six different primers, designated primer 1 to 6, were used. When the results obtained with three of the primers were combined, all five strains could be differentiated (Fig. 2). We previously analyzed the O157:H7 strains isolated in Malaysia, Japan, and Korea and EDL933 by the same AP-PCR method [13,18]. We confirmed that the AP-PCR patterns of these strains were reproducible under the present experimental condition and found that none of the five Thai strains showed an identical pattern with any of the Malaysian, Japanese, and Korean strains and EDL933 (data not shown). Other workers did not detect *E. coli* O157 in retail meats and cattle in an earlier study conducted in Thailand [7]. However, the genetic fingerprinting data suggest that the five Thai strains of *E. coli* O157 may not have been introduced recently from the neighboring area in Malaysia.

In conclusion, analysis of the sera from healthy Thai people suggested their possible exposure to *E. coli* O157 strains. This was supported by the isolation of *E. coli* strains belonging to O157:H7/H$^-$ from retail beef and bovine feces for the first time in Thailand. Although the samples were obtained in a limited geographical area in Southern Thailand in a short sampling period, genetic analysis including *stx*, plasmid, and AP-PCR profiles distinguished the five Thai strains. The results indicate a possibility that various subgroups of *E. coli* O157:H7/H$^-$ strains are distributed in Thailand. At least two strains, T1 isolated from retail beef and T13 from bovine feces, had all important virulence traits and were shown to produce Stx. Distribution of such strains in the environment is of public health significance. Clinical specimens that may be associated with *E. coli* O157:H7/H$^-$ infection need to be examined for this organism in Thailand and surrounding areas.

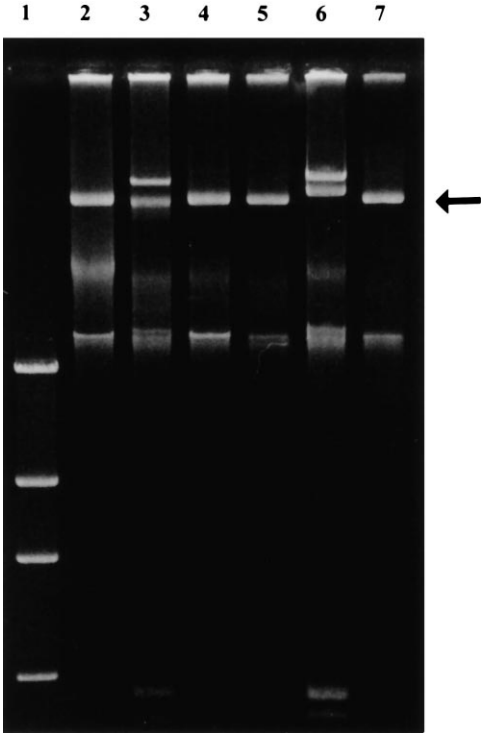

Fig. 1. Plasmid profiles of *E. coli* O157 strains. Lane 1, phage λDNA digested with *Hin*dIII; lane 2, T1; lane 3, T2; lane 4, T4; lane 5, T12; lane 6, T13; lane 7, EDL933. The 60 MDa plasmid of EDL933 is indicated by an arrow.

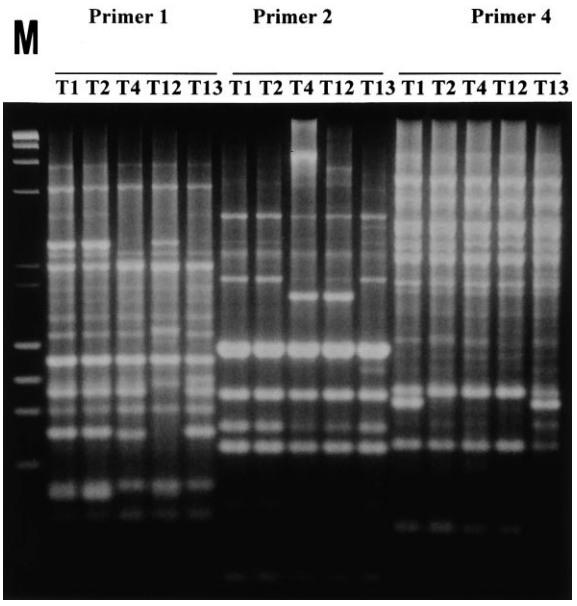

Fig. 2. Representative results of the AP-PCR analysis of *E. coli* O157 strains. The results obtained with primer 1, primer 2, and primer 4 are shown. Molecular size markers (mixture of phage λDNA digested with *Hin*dIII and phage ϕX174 DNA digested with *Hae*III) were run in the left lane (M).

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