

Molecular Typing of Enterohemorrhagic *Escherichia coli* O157: H7 Isolated in Okayama Prefecture Using Pulsed Field Gel Electrophoresis and Random Amplification of Polymorphic DNA

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Three outbreaks and many isolated cases of enterohemorrhagic *Escherichia coli* O157: H7 occurred in 1996 and 1997 in Okayama Prefecture, Japan. In an attempt to investigate the route of these infections, the strains isolated from the 3 outbreaks (total 33 strains) and 15 isolated cases (total 15 strains) were investigated using random amplification of polymorphic DNA (RAPD) and pulsed-field gel electrophoresis (PFGE). In addition, 10 strains from an outbreak in Tojo Cho, Hiroshima Prefecture (June 1996), 2 strains from the particular types of meat in Kochi Prefecture, and 42 strains isolated from bovine feces in a farm in Okayama Prefecture were also investigated in the same manner. PFGE was much more useful than RAPD for molecular typing of the clinical isolates, in that it allowed us to classify them into 10 PFGE groups. We noted that the strains differed according to the time and place of the outbreaks (or isolated cases). This indicates that O157: H7 infections in Okayama Prefecture were caused by different strains (although some cases were aggravated by the same strains as were found in other areas). The isolates from bovine feces were classified into 5 groups by PFGE profiles, but none of them were identical to those of the clinical isolates.

Key words: molecular epidemiology, enterohemorrhagic *Escherichia coli* O157: H7, pulsed field gel electrophoresis, random amplification of polymorphic DNA

and hemolytic-uraemic syndrome (HUS) among children and the elderly (1).

An outbreak of food poisoning due to EHEC O157: H7 in primary schools in Oku cho, Okayama Prefecture occurred on May 28 1996, involving 468 patients. Two of these patients died after the infection progressed to HUS. Thereafter outbreaks occurred in many places in Japan including Tojo Cho, Hiroshima Prefecture (June 11, 1996), Niimi City, Okayama Prefecture (June 16, 1996), and Okayama City (June 26, 1997), the number of patients were 185, 364 and 171, respectively (2, 3).

In these EHEC O157: H7 outbreaks, the infected food that caused the outbreak has only been identified in the following cases; Gifu Prefecture, June 1996 (vegetable salad with dried bonito shavings); Tokyo, June 1996 (beef liver); Iwate Prefecture, September 1996 (seafood salad); Hokkaido, October 1996 (potato salad); and Okayama City, June 1997 (cold Japanese noodles). Therefore, the source and infectious pattern of EHEC O157: H7 outbreaks remain obscure in most cases (4). In an attempt to clarify the source and spread pattern of EHEC O157: H7 in Okayama Prefecture, we used random amplification of polymorphic DNA (RAPD) and pulsed field gel electrophoresis (PFGE) methods with samples obtained from clinical materials. Since bovine have been implicated as a principal reservoir of EHEC O157: H7 (5-7), the O157: H7 isolates from bovine feces collected at a stock-farm in Oku Cho, Okayama Prefecture were also analyzed by the same procedures (8).

Enterohemorrhagic *Escherichia coli* (EHEC) O157: H7 is recognized as a cause of bloody diarrhea

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Materials and Methods

Origin of EHEC O157: H7 Isolates

A total of 60 O157: H7 strains obtained from patients (57 strains), foodstuffs (1 strain) and particular type of meat (2 strains) were analyzed. These were kindly provided from Okayama, Hiroshima and Kochi Prefectural public health institutes and many hospitals in Okayama Prefecture.

Of the 57 isolates gathered from patients, 8 were from isolated cases that occurred from January 1993 to May 1996 (before Oku Cho outbreak). The remaining cases occurred between July 1996 and May 1996 distributed as follows: Oku Cho outbreak, 14; Tojo Cho outbreak, 10; Niimi City outbreak, 18; isolated cases, 7. Of the 19 cases, the source of the infection had been identified by epidemiological means in an outbreak in Okayama City Hospital in June 1997.

Characterization of O157: H7 Isolates from Patients and Foodstuffs

The biochemical properties. The biochemical properties of these 61 strains were analyzed by ID test EB-20 (Nissui, Tokyo, Japan) (9).

***stx* gene profile.** Presence of the genes of Shiga-like toxin (*stx*) in each EHEC O157: H7 isolate was investigated by polymerase chain reaction (PCR) as described previously (11). Each strain was inoculated into 3 ml of Luria-Bertani (LB) broth (bacto-tryptone 1 %, bacto-yeast extract 0.5 %, NaCl 1 %) and incubated at 37°C for 12 h with shaking. After incubation, 1 ml of culture medium was centrifuged (15,000 rpm, 20 min) and resuspended in 100 μ l of distilled water. Each suspension was heated at 95°C for 5 min, immediately cooled on ice for 5 min and centrifuged at 6,000 rpm to remove debris. Four μ l of deoxynucleotide triphosphate (2.5 mM each of dATP, dCTP, dTTP, dGTP), 5 μ l of 10 \times buffer solution (20 mM Tris-HCl [pH 8.0], 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5 % Tween 20, 0.5 % Nonident P-40, 50 % Glycerol), and 5 μ l of each primer (described below) was added to each supernatant to make a final volume of 49.75 μ l.

Thereafter, 0.25 μ l of Ex-Taq containing 1.25 U (TaKaRa, Kyoto, Japan) was added. The mixture was amplified with the Gene Amp PCR system 9600 (Parkin Elmer, Norwalk, CT, USA). Three steps, denaturation at 94°C for 1 min, annealing at 62°C for 1.5 min, and extension at 72°C for 1.5 min, were repeated 30 times.

Primers to amplify the *stx-1* gene were VT-1-u (5'-GCAGTTCGTGGCAAGAGCG-3') and VT1-d (5'-GCGTCGCCAGCGCACTTG-3'). Primers to amplify the *stx-2* gene were VT2-u (5'-AATTTATATGTGGCAGGGTTC-3') and VT2-d (5'-CTTCACTGTAAATGTGTCATC-3'). The expected size of the PCR products was 522 base pairs (bp) (*stx-1*) and 806 bp (*stx-2*) (16). The products were analyzed by gel electrophoresis.

RAPD. The young cells in 4 ml of LB broth were collected by low speed centrifugation, and resuspended in 100 μ l of distilled water. The DNAs were extracted by the method of Sambrook *et al.* (10).

RAPD was carried out using the Gene Amp PCR system 9600 from Perkin Elmer. Each 25 μ l of reaction mixture contained 3 mM MgCl₂, 250 mM of each dNTP, 20 ng/ml of template DNA prepared by the method described above, primer (5'-TGCCTCGCACCA-3') (12) and 2.5 U of Taq polymerase (ampli Taq, Perkin Elmer). PCR was performed as follows: 5 min at 94°C, 5 min at 36°C, 5 min at 72°C for 4 cycles, followed by 1 min at 94°C, 1 min at 36°C, 2 min at 72°C for 30 cycles and 10 min at 72°C (12). PCR products (5 μ l) were resolved by electrophoresis through 1.8 % gels (Agarose S, Nippon Gene, Tokyo, Japan). The gels were stained with ethidium bromide (1 mg/l) and were photographed under ultraviolet (UV) transillumination.

PFGE. PFGE was performed by the method of the Japanese National Institute of Infectious Diseases (13, 14) with minor modifications. In brief, each isolated strain was grown in 5 ml of LB broth at 37°C with vigorous shaking for 12 h. After low-speed centrifugation, bacterial cells were suspended in 1 ml of distilled water, and the suspension was mixed with an equal volume of low-melting-temperature agarose (Bio-Rad Laboratories, Richmond, CA, USA). The plugs were put into appropriate lysis buffer. After lysis, the plugs were washed in Tris-EDTA (TE) buffer several times. The DNAs in the plugs were digested with 50 U of *Xba* I (Toyobo, Osaka, Japan) at 37°C for 24 h. PFGE was performed with a 1 % agarose gel by CHEF DR II (Bio-Rad Laboratories) in 0.5 % Tris-borate-EDTA (TBE) buffer at 12°C and 200 V. Linearly ramped switching time was divided into two stages; the first stage was performed from 4 to 8 sec for 12 h, and the second stage was from 8 to 50 sec for 10 h. These stages were necessary to separate each digested fragment of the genome.

After PFGE, the gel was stained with 1 mg/l eth-

Table 1 Properties of the strains isolated from 1993 to 1998

Date of onset	Place	Origin	Source	Number of strains	Presence of genes		Groups		Cluster
					<i>stx-1</i>	<i>stx-2</i>	RAPD	PFGE	
Jan. '93	Kurashiki City	Patient	Kurashiki Central Hp	1	—	+	A	1	a
Jul. '93	Kurashiki City	Patient	Kurashiki Central Hp	1	+	+	A	2	b
Jul. '93	Kurashiki City	Patient	Kurashiki Central Hp	1	+	+	A	—	—
Jan. '94	Takahashi City	Patient	Kurashiki Central Hp	1	+	+	A	3	c
Jun. '94	Kurashiki City	Patient	Kurashiki Central Hp	1	+	+	A	4	c
Jul. '94	Kurashiki City	Patient	Kurashiki Central Hp	1	+	+	A	4	c
Aug. '94	Soja City	Patient	Kurashiki Central Hp	1	+	+	B	—	—
May '96	Kurashiki City	Patient	Kurashiki Central Hp	1	+	+	A	5	b
May '96	Oku Cho outbreak	Patients	Okayama PHI	14	+	+	A	5	d
Jun. '96	Tojo Cho outbreak	Patients	Hiroshima PHI	10	+	+	A	6	d
Jun. '96	Niimi City outbreak	Patients	Okayama PHI	18	+	+	A	7	d
Aug. '96	Kurashiki City	Patient	General practitioner	1	+	+	A	8	e
Aug. '96	Kochi Prefecture	Particular type of meat	Kochi PHI	2	+	+	A	8	e
Oct. '96	Wake Cho	Patient	General practitioner	1	+	+	A	9	f
Nov. '96	Tsuyama City	Patient	Tsuyama Central Hp	1	+	+	A	10	g
Nov. '96	Kurashiki City	Patient	General practitioner	1	+	+	A	—	—
Feb. '97	Katsuyama Cho	Patient	General practitioner	1	+	+	A	9	f
May '97	Okayama City	Patient	Sato Hp	1	+	+	A	4	c
Jun. '97	Okayama City outbreak	Food	Okayama PHI	1	+	+	A	6	d
May '98	Okayama City	Patient	Okayama National Hp	1	—	+	A	1	a

60

RAPD: Random amplification of polymorphic DNA; PFGE: Pulsed field gel electrophoresis; —: Pattern analysis was impossible due to broad ladder; PHI: Prefectural public health institute; Hp: Hospital

idium bromide and was photographed under UV transillumination.

Isolation of O157: H7 from Bovine Feces

We tried to isolate EHEC O157: H7 from fresh bovine feces in kind cooperation with a farm in Okayama Prefecture. Each fecal sample was collected with Seed Swab-1 (Eiken, Tokyo, Japan) from each bovine immediately after defecation. Forty samples were collected in August of 1996 and 10 samples in August of 1997. The samples collected were stored at -70°C until the following analysis.

Screening PCR was performed to detect the *stx* gene. Approximate $10\mu\text{l}$ of each fecal sample was added to 3 ml of LB broth and incubated at 37°C for 12 h with shaking. Thereafter, these preparations were checked by PCR as described above using the common primers which detect both *stx-1* and *stx-2* genes; VT com-u (5'-GAGCGAAATAATTTATATGTG-3') and VT com-d (5'-TGATGATGGCAATTCACCTAT-3') (16). O157: H7 cells were then isolated from PCR positive feces

preparations by the method of Immuno Magnetic Separation (IMS). Incubated culture medium (1 ml) in a 1.5 ml tube was first added to $20\mu\text{l}$ of magnetic beads suspension (Dynabeads anti-*E. coli* O157, Dynal, Oslo, Norway), and then incubated for 30 min at room temperature (15). The bead pellets were resuspended in $100\mu\text{l}$ of TE buffer. After inoculation with $50\mu\text{l}$ of resuspension on Sorbitol MacKonkey agar plate, the plates were incubated at 37°C for 12 h. Forty non-sorbitol-fermenting colonies were identified by ID test EB-20 (Nissui) (9). Strains identified as *E. coli* were confirmed by agglutination with both *E. coli* O157 and H7 antisera (Denka Seiken, Tokyo, Japan). The strains were analyzed by PCR with *stx-1* and *stx-2* primers and then by PFGE.

Results

Characterization of O157: H7 Isolates from Patients and Foods

Fifty-eight strains of O157: H7 isolated from food

poisoning patients and 2 from contaminated beef were studied. The date of onset, the place where the food poisoning occurred, and the origin and source of the strains are summarized in Table 1.

Biochemical properties. All 60 strains showed the same biochemical properties on EB-20: Production of H₂S (-), escrine (-), PPA (phenylpyruvic acid) (-), Indole (+), lysine decarboxylase (+), arginine decarboxylase (-), ornithine decarboxylase (+), urease (-); use of citric acid (-), malonic acid (-), adonitol (-), inositol (-); fermentation of raffinose (+), rhamnose (+), sorbitol (-), sucrose (+), mannitol (+) and arabinose (+); VP (Voges-Proskauer) test (-), ONPG (o-nitrophenyl- β -D-galactopyranoside) test (+).

stx gene profiles. The presence or absence of *stx-1* and *stx-2* genes were determined by PCR using the specific primers for these genes. Two out of 60 had only the *stx-2* gene but the other 58 strains had both *stx-1* and *stx-2* genes.

RAPD profiles. On RAPD analysis, 59 isolates showed the same profile (group A), but an isolated case in Soja City (August 1994) revealed a slightly different pattern (group B) (Fig. 1, Table 1).

PFGE profiles. The 60 isolates were classified into 10 groups (groups 1 to 10) based on their PFGE profiles (Fig. 2). EHEC O157: H7 strains showing the same PFGE profile were consistently isolated within each major outbreak, 14 strains from the Oku Cho outbreak (group 5), 10 strains from the Tojo Outbreak (group 6) and 18 strains from the Niimi City outbreak (group 7), and the profiles of these three PFGE groups were closely related (Fig. 2, lanes 17-19). Since the profiles of strains belonging to group 3 and 4 were also similar, these 10 groups could be classified into 7 clusters (Figs. 2 and 3, Tables 1 and 2). The geographic distribution of the isolates in distinct PFGE groups is shown in Fig. 4.

Characterization of EHEC O157: H7 Isolates from Bovine Feces

On the screening PCR using *stx* gene primers, the rate of *stx* gene positive bovine feces was 10/40 (25%) in 1996 and 10/13 (77%) in 1997. Isolation of EHEC O157: H7 from the *stx*-positive feces was performed by IMS. No strain was isolated from the feces in 1996, whereas a total of 42 isolates were obtained from 10 feces samples in 1997. The results of biochemical tests on all 42 isolates were identical to those of the clinical isolates. On PCR with common primers for *stx-1* and *stx-2* genes, only 806 bp was produced in all 42 isolates, indicating that

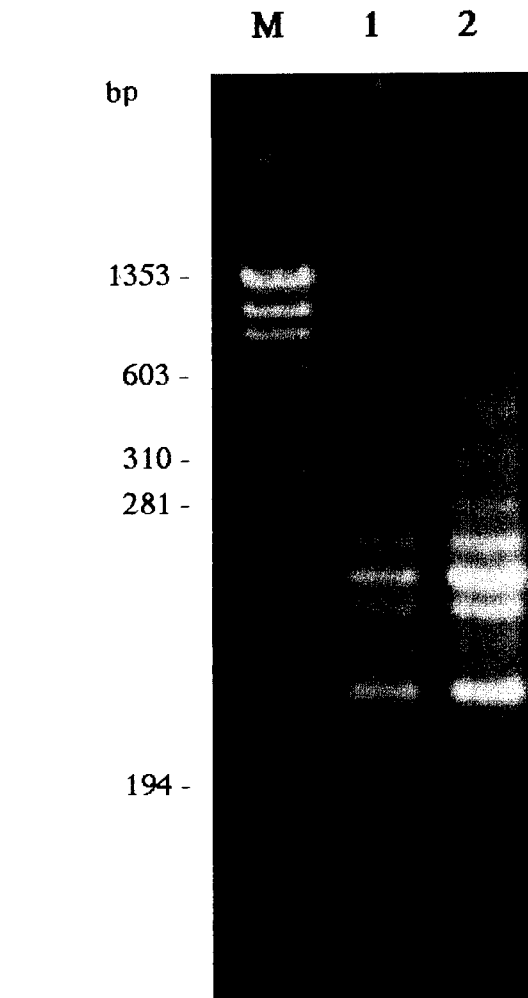


Fig. 1 Two representative patterns of random amplification of polymorphic DNA (RAPD). The RAPD pattern shown in lane 1 was observed in an isolated case in Soja City (Aug. 1994). M: Molecular size marker (ϕ x174/*Hae* III digest); bp: Base pair.

these strains have only the *stx-2* gene. The isolate were classified into 5 groups (groups I to V) based on PFGE profiles (Table 2, Fig. 2), but the resulting profiles were quite different from those of clinical isolates.

Discussion

PFGE has been widely used as a DNA fingerprinting method and has been shown to have advantages over other DNA-based methods such as RFLP, ribotyping, and RAPD (19). We analyzed 60 EHEC O157: H7 strains by RAPD and PFGE as well as their biochemical properties and distribution of *stx-1* and *stx-2* genes. Few

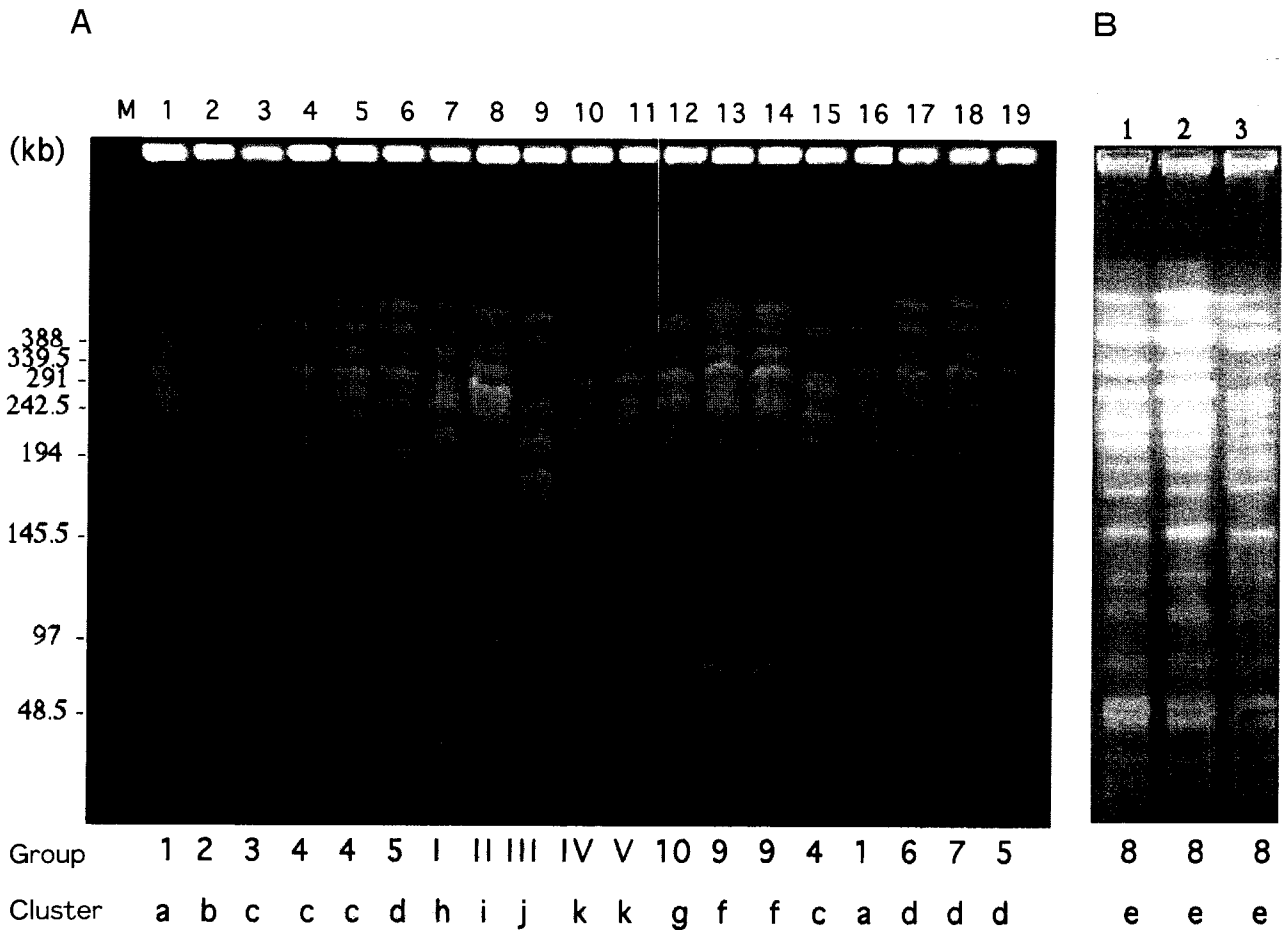


Fig. 2 Representative patterns of 10 pulsed field gel electrophoresis (PFGE) groups (groups I–10, as indicated at the bottom of the figure) from clinical isolates and 5 PFGE groups (groups I–V) from cattle feces.

A: M, Molecular size marker (48.5-Kb λ DNA ladder); 1, January 1993 Kurashiki City; 2, July 1993 Kurashiki City; 3, January 1994 Takahashi City; 4, June 1994 Kurashiki City; 5, July 1994 Kurashiki City; 6, May 21, 1996 Kurashiki City; 7–11, August 1997 Okayama Prefecture bovine feces; 12, November 1996 Tsuyama City; 13, October 1996 Wake Cho; 14, February 1997 Katsuyama Cho; 15, June 1997 Okayama City; 16, May 1998 Okayama City; 17, June 1996 Tojo Cho outbreak and June 1997 Okayama City outbreak; 18, June 1996 Niimi City outbreak; 19, May 28 1996 Oku Cho outbreak **B:** 1, August 1996 Kurashiki City; 2–3, August 1996 Kochi Prefecture particular type of meat

differences were observed regarding on their biochemical properties, distribution of toxin genes and RAPD profiles. However, they could be classified into 10 different groups by PFGE profiles. Hunter *et al.* reported that PFGE is a much better procedure than RAPD for epidemiological studies investigating the diversity of the strains (19). Simpson's Diversity values for RAPD and PFGE are 0.033 and 0.790, respectively. This was confirmed in our study, too, and we concluded that the infections of EHEC O157: H7 in Okayama Prefecture were caused by several different strains, although some cases were aggravated by the same strains as occurred in

other places.

The PFGE profiles of the strains isolated from the Okayama City outbreak (June 1997) and the Tojo Cho outbreak (June 1996) were identical (20), and very closely related to those of the Oku Cho outbreak (May 1996) and the Niimi City outbreak (June 1996) as reported by Izumiya *et al.* (14), but different from the strains isolated from isolated cases. These data suggest that these might be the strains which readily cause outbreaks. We are now investigating the detailed biochemical properties of these strains to answer this question.

The PFGE profiles of the strains isolated from the

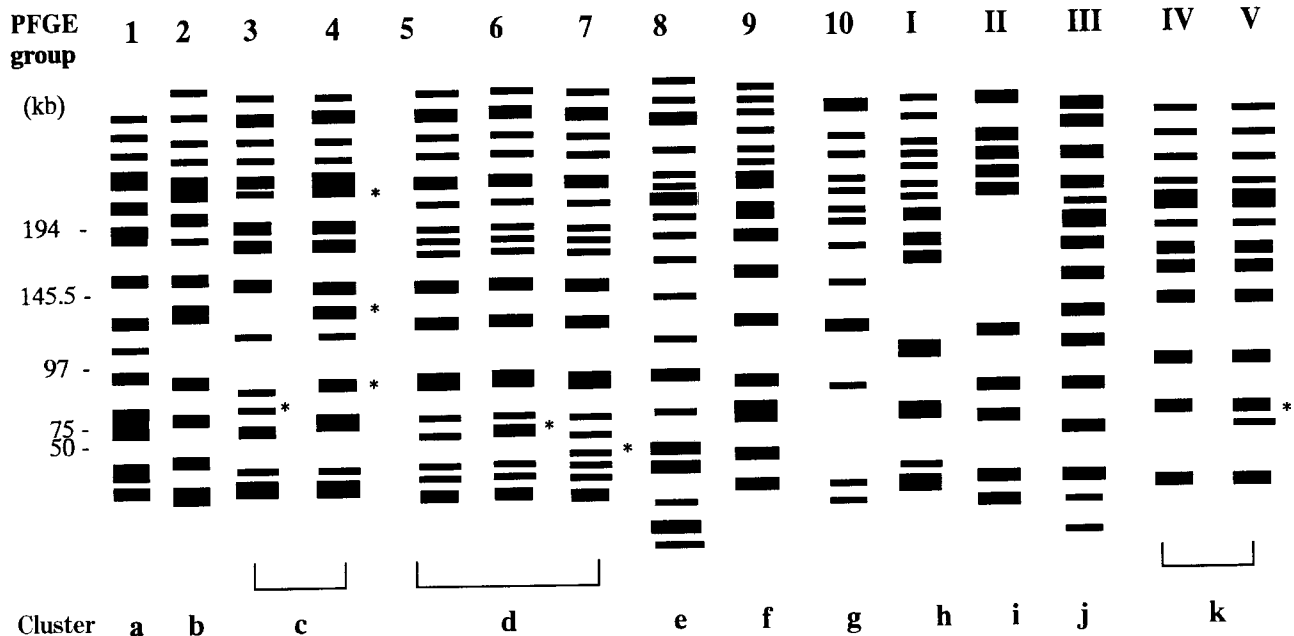


Fig. 3 Schematic diagram of 10 pulsed field gel electrophoresis (PFGE) groups (groups 1-10) from clinical isolates and 5 PFGE groups (groups I-V) from bovine feces, with asterisks (*) on the right side of marker bands for grouping. These 15 PFGE groups were classified into 11 clusters (a-k, as indicated at the bottom) based on similarity of the patterns. Molecular size markers are shown to the left.

Table 2 Properties of the strains isolated from cattle feces in 1997

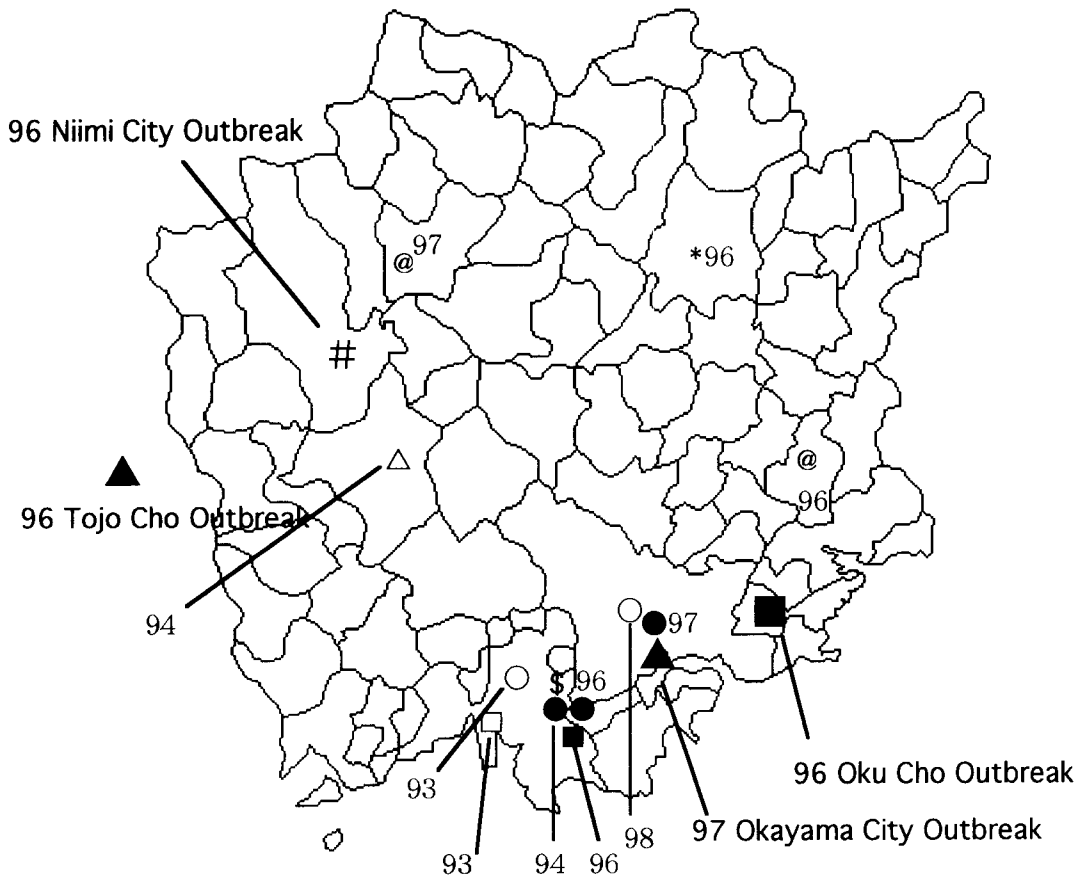
Cow	Number of strains isolated	PFGE groups (Number of strains)	Cluster
A	1	I(1)	h
B	1	I(1)	h
C	4	I(4)	h
D	4	I(2), II(2)	h, i
E	1	II(1)	i
F	4	II(4)	i
G	5	II(5)	i
H	1	III(1)	j
I	10	II(4), IV(2), V(4)	i, k
J	11	V(11)	k
42			

A total of 42 strains were isolated from 10 bovine designated A to J. The isolates were classified into 5 groups (group I to V) based on their pulsed field gel electrophoresis (PFGE) profiles.

Oku Cho outbreak (May 28, 1996) and a strain from an isolated case in Kurashiki City (May 21, 1996) were identical (group 5 in Table 1 and Figs. 2 and 3), indicating that the causative strain of the Oku Cho outbreak had already been in existence in Okayama Prefecture before

the outbreak. The PFGE profiles of a strain isolated in Kurashiki City (August 1996) and 2 strains from particular type of beef meat in Kochi Prefecture (August 1996), group 8, were identical, suggesting that particular type of meat may be one of the main causative agents in O157:H7 infection as has already been pointed out (4).

Both Oku Cho and Niimi City are located in rural areas. We tried to isolate the EHEC O157:H7 strains from cattle feces obtained from a farm in Oku Cho, in order to investigate their PFGE profiles. The positive rates of the bovine having the *stx* gene were 25% (1996) and 77% (1997). These rates are much higher than those reported previously (0.30-0.397%) (18). From 10 bovine (feces samples), no EHEC O157:H7 cells were isolated in 1996 (probably because of our poor technique), whereas 42 EHEC O157:H7 strains were isolated in profiles quite different from clinical isolates. We are now working on fecal samples obtained from other stock farms in Okayama Prefecture to study whether this particular farm was exceptionally contaminated or not. From these samples we are also trying to isolate a strain of EHEC O157:H7, the PFGE profiles of which are identical to those of clinical isolates.



PFGE groups (mark)	Isolates (Number of strains)
1 (○)	Jan.'93: Kurashiki City (1), May '98: Okayama City (1)
2 (□)	Jul.'93: Kurashiki City (1)
3 (△)	Jan.'94 : Takahashi City (1)
4 (●)	Jun. '94 : Kurashiki City (1), Jul.'94: Kurashiki City (1) May '97: Okayama City (1)
5 (■)	May '96: Kurashiki City (1) and Oku Cho outbreak (14)
6 (▲)	Jun. '96 : Tojo Cho (Hiroshima Pref.) outbreak (10), Jun. '97: Okayama City outbreak (1)
7 (#)	Jun. '96: Niimi City outbreak (18)
8 (\$)	Aug. '96: Kurashiki City (1) and Kochi Pref. particular type of meat(2)
9 (@)	Oct. '96: Wake Cho (1) : Feb. '97: Katsuyama Cho (1)
10 (*)	Nov. '96: Tsuyama City (1)

Fig. 4 The map of Okayama Prefecture and distribution of the clinical isolates in distinct pulsed field gel electrophoresis (PFGE) groups

Acknowledgments. The authors would like to thank Dr. Mutsuo Tanaka (Kurashiki Central Hospital), Dr. Taiji Kunitomi (Okayama Red Cross Hospital), and Dr. Yozo Ichiba (Okayama National Hospital) for their kind cooperation and support.

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Received March 10, 1999; accepted March 25, 1999.