Molecular epidemiological study on enterohemorrhagic *Escherichia coli* O157:H7 isolates from cattle using pulsed-field gel electrophoresis

(パルスフィールドゲル電気泳動を用いた牛由来腸管出血性大腸菌 O157:H7 に関する分子疫学的研究)

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Contents

Abbreviations

A/E : attaching and effacing

BFP : bundle-forming pilus

bp : base pair

CDC : Centers for Disease Control and Prevention

CFAs : colonizing factor antigens

DIG : digoxigenin

E. coli : *Escherichia coli*

EDTA : ethylenediaminetetraacetic acid

EET : 100 mM EDTA, 10 mM EGTA, 10 mM Tris pH 8.0

EET-LS : EET with 200 μ g/ml lysozyme and 0.05 % N- laurylsarcosine sodium salt

EET-SP : EET with 1 mg/ml proteinase K and 1.0 % (W/V) lauryl sulfate sodium

EGTA : [ethylene-bis(oxyetylenenitrilo)] tetraacetic acid; egatazic acid

EHEC : enterohemorrhagic *E. coli*

EIEC : enteroinvasive *E. coli*

EPEC : enteropathogenic *E. coli*

ESP : EPEC secreted protein

ETEC : enterotoxigenic *E. coli*

Gb_3 : globotriaocylceramide

HC : hemorrhagic colitis

HUS : hemolytic uremic syndrome

IMS : immunomagnetic separation

Ipa : invasion plasmid antigen

kb : kilobase

LEE : locus of enterocyte effacement

LT : heat-labile toxin

MPN : most probable number

NIID : National Institute of Infectious Diseases

OMP : outer membrane protein

PCR : polymerase chain reaction

PFGE : pulsed-field gel electrophoresis

RFLP : restriction fragment length polymorphism

SDS : sodium dodecyl sulfate

sen : Shigella enterotoxin

SMAC-CT : MacConkey sorbitol agar plates with 50 ng/ml cefixime and 2.5 μ g/ml tellurite

SPIPHE : Shizuoka Prefectural Institute of Public Health and Environmental Science

ST : heat-stable toxin

STEC : Shiga toxin-producing *E. coli*

Stx : Shiga toxin

TBE : 89 mM Tris, 89 mM boric acid, 2 mM EDTA

TE : 10 mM Tris, 1 mM EDTA pH 8.0

Tir : translocated intimin receptor

Tris : tris (hydroxymetyl) aminomethane

U : units

UPGMA : unweighted pair group method using arithmetic average

VTEC : verotoxigenic *E. coli* or Verocytotoxin-producing *E. coli*

Preface

Escherichia coli (*E. coli*) is one of the normal flora of warm-blooded animals and occurs in the lower part of the intestine (14). The organism belongs to the family Enterobacteriaceae and is facultatively anaerobic, motile or nonmotile, gram negative and straight rods. Most strains of *E. coli* are Indole positive, β -glucuronidase positive, Voges-Proskauer negative, citrate negative, and malonate negative, and most ferment lactose and glucose rapidly. A majority of *E. coli* strains are also negative for H_2S and urease production (14). *E. coli* is subdivided serologically. Though the complete serotyping includes somatic (O), capsular (K), flagellar (H), and fimbrial (F) antigens, *E. coli* strains are generally described in combination with O and H antigens, such as O1:H1. A total of 166 different O antigens and 52 different H antigens have been listed in the *E. coli* antigenic scheme (29). A specific combination of O and H antigens defines the "serotype" of an isolate.

Some groups of *E. coli* cause enteric/diarrheal disease and are distinguished from general *E. coli* strains. Diarrheagenic *E. coli* strains are classified as enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), and so forth, on the basis of pathogenic features (36). Table 1 shows the characteristics of major diarrheagenic *E. coli* categories. The term EHEC was originally coined to denote strains that cause hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) in humans. HC is characterized by severe crampy abdominal pain, watery diarrhea followed by grossly bloody diarrhea, and little or no fever. On the other hand, HUS which is defined by the triad of acute renal failure, thrombocytopenia, and microangiopathic hemolytic anemia, is typically preceded by a bloody diarrheal disease indistinguishable from HC (36).

Karmali *et al.* (20) reported an association between sporadic cases of HUS and fecal cytotoxin and cytotoxin-producing *E. coli* in stools in 1983. The cytotoxin assay used by Karmali et al. was originally reported by Konowalchuck *et al.* (25) in 1977. They reported that culture filtrates from some strains of *E. coli* produced a cytopathic effect on cultured Vero cells. The term "verotoxigenic *E. coli*" or "Verocytotoxin-producing *E. coli*" (VTEC) was derived from this observation. An alternative nomenclature is "Shiga toxin-producing *E. coli*" (STEC), which reflects the fact that one of the

Table 1. Characteristics of the diarrheagenic *E. coli* categories

Category	Serotype	Virulence factor	Colinization site
ETEC: Enterotoxigenic *E. coli*	O6:H16(H−), O8:H9(H−) O25:H−, O27:H7 O126:H12, O128:H12 O148:H28, O159:H−	Fimbriae (CFAs) Enterotoxin (LT, ST)	Small intestine
EPEC: Enteropathogenic *E. coli*	O18:H−, O55:H6(H−) O86:H27, O111:H− O119:H6, O142:H6 O165:H−, O167:H−	Fimbriae (BFP) TypeⅢ secretion system Secreted proteins (Esp proteins) (Esp proteins) intimin	Small intestine
EHEC: Enterohemorrhagic *E. coli*	O26:H11, O55: H7 O103: H2, O111:H8(H−) O113:H21, O145:H− O157:H7	Shiga toxins (Stx) TypeⅢ secretion system Secreted proteins (Esp proteins) intimin Enterohemolysin	Large intestine
EIEC: Enteroinvasive *E. coli*	O28:H−, O29:H− O124:H−, O136: H− O143:H−, O144:H− O164:H−, O173:H−	TypeⅢ secretion system Secreted proteins (Ipa proteins) Enterotoxin (*sen*)	Large intestine

cytotoxins produced by these organisms is essentially identical at the genetic and protein levels to the Shiga toxin (Stx) produced by *Shigella dysenteriae* 1 (39). VTEC and STEC are equivalent terms, and both refer to *E. coli* strains that produce one or more toxins of the Stx family. EHEC refers to *E. coli* strains that produce Stx and hemolysin (enterohemolysin) and cause attaching and effacing (A/E) lesions on epithelial cells. Whereas not all STEC strains are believed to be pathogens, all EHEC strains are considered pathogens. *E. coli* O157:H7 strains belong to the EHEC category (36).

The major virulence factor of EHEC is Stx. The Stx family contains two major, immunologically non-cross-reactive groups called Stx1 and Stx2. A single EHEC strain is known to produce Stx1 only, Stx2 only, or both toxins. Stx1 from EHEC is identical to Stx from *S. dysenteriae* 1. While Stx1 is highly conserved, sequence variation does exist within Stx2. The basic A-B subunit structure is conserved across all members of the Stx family. The B subunit binds the Stx to a specific glycolipid receptor, globotriaosylceramide or Gb_3, which is present on the surface of eukaryotic cells. The A subunit is subsequently translocated to the cytoplasm. Of the A subunit, A_1 peptide is an N-glycosidase that removes a single adenine residue from the 28s rRNA of eukaryotic ribosomes, thereby inhibiting protein synthesis. The disruption of protein synthesis leads to the death of renal endothelial cells, intestinal epithelial cells, or Vero cells, which possess the Gb_3 receptor (36).

The locus of enterocyte effacement (LEE) pathogenicity island encoded type III secretion apparatus, secreted proteins, and intimin involve A/E lesion formation in the large intestine. The interaction between 94- to 97-kDa outer membrane protein (OMP) called intimin and the secreted protein called Tir (translocated intimin receptor) contributes to the intimate adherence of EHEC to epithelial cells and produces a microvillous effacement (10).

Most EHEC O157:H7 strains harbor an approximately 90 kilobase (kb) plasmid that contains genes encoding a hemolysin (enterohemolysin). Enterohemolysin is found in almost all EHEC O157:H7 strains and is widely distributed among other STEC. The role of enterohemolysin is still unclear. Lysis of erythrocytes *in vivo* releases heme and hemoglobin, which enhance the growth of EHEC O157:H7 and may serve as a source of iron (36).

The first outbreak of EHEC O157:H7 was reported in 1982 by Riley *et al.* (44). They showed that the two outbreaks were associated with undercooked hamburgers at the same fast food restaurant chain. An *E. coli* serotype O157:H7, which had not previously been recognized as a pathogen, was isolated from stools collected from the victims of the outbreaks. Since then, isolation of this organism has increased exponentially, and the organism has emerged as a public health concern in North America and Europe (2). The Centers for Disease Control and Prevention (CDC) in the United States estimates the annual disease burden of EHEC O157:H7 in the United States to be more than 20,000 infections and as many as 250 deaths (8).

In Japan, since the first outbreak of this pathogen in 1990 in a kindergarten in Urawa City, the number of isolations by prefectural and municipal public health institutes has been increasing slightly from year to year (37). In 1996, multiple outbreaks of and sporadic infections with EHEC O157:H7 occurred in many places in Japan (38). Table 2 shows an outline of major outbreaks in 1996 involving more than ten symptomatic patients. Five thousand, seven hundred and twenty-seven symptomatic patients were recorded in an apparent single outbreak among school children in Sakai City, and 3 of these patients died. In total, 9,451 symptomatic patients, 1,808 hospitalized patients, and 12 deaths were reported to the Ministry of Health and Welfare of Japan in 1996 (38). Since then, infection with EHEC O157:H7 has become a matter of concern that should be prioritized in Japan.

Ground beef was identified as the vehicle of this pathogen in 58% of foodborne outbreaks reported to the CDC in the United States up to 1994 (2). EHEC O157:H7 has been isolated from the feces of 1-10% of sampled cattle in surveys in North America and Europe (2). The agent appears to be virtually ubiquitous on cattle farms (13). Therefore, cattle are suspected to be one of the most significant sources of EHEC O157:H7 in North America and Europe. On the other hand, EHEC O157:H7 was also isolated from salads in three of the 16 outbreaks in 1996 in Japan (Table 2). The consumption of white radish sprouts was also associated with two of the 16 outbreaks (Table 2) (32, 49). None of the specific food ingredients were identified in the remaining outbreaks in 1996. Though some sporadic infections were linked to the consumption of beef or cattle liver (18, 38), the importance of cattle and cattle farms as a source of all the human EHEC O157:H7 infections was not recognized in Japan at

Table 2. EHEC O157:H7 outbreaks[a] in Japan, 1996 and the characteristics of the isolates

Place	Facilities	Month	Symptomatic patients	Death	Contaminated foods	Chracteristics of the isolates		
						stx1	stx2	PFGE type[b]
Okayama (Oku)	Primary school	May–Jun	468	2		+	+	I c
Gifu (Gifu)	Primary school	Jun	379		Salads[c]	+	+	I b
Hiroshima (Tojo)	Primary school	Jun	185			+	+	I a
Aichi (Kasugai)	Camp school	Jun	21			+	+	I b
Fukuoka (Fukuoka)	Day-care center	Jun	48			+	+	I a
Okayama (Niimi)	Primary school	Jun	364			+	+	I b
Osaka (Kawachinagano)	Day-care center	Jun	49			+	+	I b
Tokyo (Minato-ku)	Box lunch shop	Jun	191			+	+	II d
Gunma (Sakai)	Primary school	Jun–Jul	138			−	+	IV
Osaka (Sakai)	Primary school	Jul	5,727	3	White radish sprouts[d]	+	+	II a
Osaka (Habikino)	Old-age home	Jul	98			+	+	II a
Kyoto (Kyoto)	Restaurant	Jul	74	1	White radish sprouts[d]	+	+	II a
Wakayama (Hashimoto)	Old-age home	Jul	16			+	+	II a
Wakayama (Gobo)	Old-age home	Jul–Aug	20			+	+	II a
Iwate (Morioka)	Primary school	Sep	47		Salads, Seafood sources[c]	+	+	II j
Hokkaido (Obihiro)	Kindergarten	Oct	157		Salads[c]	−	+	III h

[a] The number of the symptomatic patients is more than 10.

[b] The classification by the National Institute of Health of Japan (16).

[c] EHEC O157:H7 was recovered.

[d] The epidemiological investigations demonstrate the association between the outbreaks and consumption of white radish sprouts (32, 49).

that time.

In Japan, EHEC O157:H7 was first isolated from calf with diarrhea in 1981 (34). There was little information on the prevalence of EHEC O157:H7 among cattle populations throughout Japan before the multiple outbreaks occurred in 1996. The Ministry of Agriculture, Forestry and Fisheries of Japan conducted on-farm surveys to evaluate the prevalence of this agent from August to October of 1996. EHEC O157:H7 was isolated from 0.62% of cattle fecal samples but not from any of the samples taken from pigs (45). In addition, EHEC O157:H7 was isolated from 1.4% of cattle fecal samples and 0.3% of swab samples from cattle carcasses in abattoirs according to a survey conducted by the Ministry of Health and Welfare of Japan from July to September of 1996 (45). However the ecology and mode of transmission of this agent on cattle farms are still unclear. In consequence, the importance of cattle populations as a possible source of human EHEC O157:H7 infections is also unexplained to date.

One of the goals of epidemiology is to understand the ecology and mode of transmission of a causative agent based on its identification and discrimination. A comprehension of epidemiology of a pathogen leads to effective measures against the agent. In late years, an identification and discrimination of a pathogen by molecular biological methods has become popular in addition to the conventional methods based on the phenotypes of an agent such as the metabolic features and the immunological characteristics of the surface antigens. The epidemiological analysis based on the genotypes of an agent is called the molecular epidemiology. Pulsed-field gel electrophoresis (PFGE) is one of the most general techniques in molecular epidemiological analysis (3, 6, 16, 26, 29, 31).

In order to gain insight into the importance of cattle populations as a possible source of human EHEC O157:H7 infections in Japan, a series of epidemiological studies were performed. First of all, an evaluation of some epidemiological markers including PFGE was performed to find the best method for discrimination of this organism. Subsequently, the duration and magnitude of fecal shedding of EHEC O157:H7 from naturally and experimentally infected cattle was investigated. The genotype of the EHEC O157:H7 isolates defined by PFGE was also investigated. Finally, EHEC O157:H7 isolates from cattle in Japan and the USA were compared by using molecular biological methods to discuss the epidemiology of this agent from a global viewpoint.

Chapter I

Molecular typing of enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 (H-) isolates from cattle in Japan

Introduction

EHEC O157:H7 was first identified in an outbreak in the United States in 1982 (44). Ground beef was identified as the vehicle of this pathogen in 58% of the foodborne outbreaks reported to the CDC in the United States through 1994 (2). EHEC O157: H7 has been isolated from the feces of 1 - 10% of sampled cattle in surveys in North America and Europe (2). The agent appears to be virtually ubiquitous on cattle farms (13). Therefore cattle are suspected to be one of the most important sources of EHEC O157:H7 in North America and Europe (2).

In Japan, since the first outbreak of this pathogen in a kindergarten in Urawa City, 1990, the number of isolations by prefectural and municipal public health institutes has increased slightly (37). In 1996, multiple outbreaks and sporadic infections of EHEC O157:H7 occurred in many places in Japan (38). However, products of bovine origin have neither been confirmed nor suspected as the vehicle for EHEC O157:H7 outbreaks in that year in Japan (38). According to the results of surveys conducted by Japanese government in 1996, EHEC O157:H7 was isolated from less than 1.5% of cattle in Japan. However the ecology and mode of transmission of this agent on cattle farms is still unclear in Japan.

In order to conduct an epidemiological study of EHEC O157:H7 among cattle populations, suitable epidemiological marker for discrimination of the isolates needed to be found. This chapter addresses the results of evaluations of epidemiological markers, i.e., toxin profile, phage type, and PFGE, as a method of subtyping EHEC O157:H7 isolates from cattle in Japan. In addition, the results of a comparison of these data with existing data on human isolates are also presented to discuss the importance of cattle population in Japan as a possible source of human EHEC O157:H7 infections.

Materials and Methods

Strains

Sixty-nine EHEC O157:H7 isolates and eight EHEC O157:H- isolates from 77 bovine fecal samples were isolated at regional livestock hygiene service centers of 23 prefectures in Japan from August to October in 1996 (Table 3). Eleven isolates from patients in 1996 were kindly provided by the National Institute of Infectious Diseases (NIID) of Japan and were used as reference strains (Table 4). These human isolates possesed a variety of PFGE patterns belonging to types I a, I b, I c, II a, III a, III b, III c, III d, III e, IV, V a (classification by NIID of Japan) (16).

Toxin profile

The presence of the *stx* genes of each EHEC O157 isolate was deterimined by multiplex polymerase chain reaction (PCR) using the methods of Kobayashi (23). Briefly, a primer pair V1 (5' AGTTAATGTGGTGGCGAA) and V5 (5' GACTCTTCCATCTGCCG) was used to amplify a 811 base pair (bp) fragment from the *stx1* gene. A primer pair V3 (5' TTCGGTATCCTATTCCCG) and V4 (5' TCTCTGGTCATTGTATTA) was used to amplify a 471 bp DNA fragment from the *stx2* gene. Each PCR was performed in a volume of 50 μl containing 200 μM of dNTP mixture (Takara Shuzo Co., Kyoto, Japan), 2 μl of template, 1.25 units (U) of Taq DNA polymerase (Takara Shuzo Co.), and different concentrations of each primer set. The concentration of the primar pairs were 20 pM for *stx1* gene and 100 pM for *stx2* gene. Each PCR cycle consisted of 30 s at 94 ℃, followed by 30 s at 55 ℃ and 30 s at 72 ℃ in a TaKaRa PCR Thermal Cycler TP2000 (Takara Shuzo Co.). In total, 25 cycles were performed for each reaction. PCR products were analyzed by electrophoresis in 2% agarose (Wako Pure Chemical Industries, Osaka, Japan) and stained with ethidium bromide. pHY markers (Takara Shuzo Co.) were used as size markers.

Phage typing

Phage typing was performed by the methods orginally described by Ahmed *et al.* (1) and extended by Khakhria *et al.* (22). Shizuoka Prefectural Institute of Public Health

Table 3. Results of the typing of *E. coli* O157 isolates from cattle in Japan

No.	Origin[a]	H antigen	*stx1*	*stx2*	PFGE type[b]	Phage type
1	A	7	−	+	1	34
2	B	7	−	+	2	31
3	C	−	−	+	3	34
4	D	7	−	+	4	32
5	E	7	−	+	5	54
6	E	7	−	+	5	54
7	E	7	−	+	6	74
8	F	7	+	+	7 (Ⅰa)[c]	32
9	F	7	+	+	7 (Ⅰa)	21
10	A	7	−	+	8	32
11	A	7	+	−	9	4
12	G	7	+	+	10	54
13	H	7	+	+	11	54
14	H	7	−	+	12	54
15	I	7	−	+	13	8
16	I	7	−	+	13	8
17	I	7	−	+	13	8
18	I	−	−	+	14	54
19	J	7	−	−	15	34
20	J	7	−	+	16	32
21	J	7	−	+	17	32
22	K	7	−	+	18	39
23	K	7	−	+	18	39
24	K	7	−	+	18	39
25	L	7	−	+	19	70
26	L	7	+	+	20	14
27	L	−	−	+	21	31
28	C	−	−	+	3	54
29	C	7	+	+	22	54
30	M	7	+	+	23 (Ⅱa)	21
31	M	7	+	−	24	54
32	M	7	+	−	24	54
33	M	7	−	+	25	54
34	M	7	+	+	23 (Ⅱa)	21
35	M	7	+	+	26	21
36	M	7	+	+	26	21
37	M	7	+	+	27	21
38	M	7	+	+	26	40
39	M	7	+	+	26	21
40	M	7	+	+	26	21
41	M	7	+	+	26	21
42	M	7	+	+	28	21
43	M	7	+	+	26	40
44	M	7	+	+	29	21
45	M	7	+	+	26	21

Continued on following page

Table 3-*Continued*

No.	Origin[a]	H antigen	*stx1*	*stx2*	PFGE type[b]	Phage type
46	M	7	+	+	26	21
47	M	7	−	+	30	8
48	M	7	−	+	31	34
49	M	7	−	+	32	34
50	M	7	−	+	33	54
51	M	7	+	−	34	54
52	M	7	−	+	32	34
53	M	7	+	+		21
54	M	7	+	+	35 (I c)	21
55	M	7	+	+	35 (I c)	21
56	N	7	−	+	36	8
57	O	7	−	+	37	8
58	P	−	−	+	38	2
59	P	−	−	+	38	2
60	P	−	−	+	38	2
61	P	−	−	+	38	2
62	Q	7	−	+	39	34
63	C	7	+	+	22	54
64	R	7	−	+	40	71
65	R	7	−	+	40	54
66	R	7	−	+	41	71
67	R	7	−	+	40	71
68	R	7	+	+	42	1
69	S	7	−	+	43	34
70	T	7	+	+	42	1
71	T	7	+	+	44	1
72	L	7	−	+	45	70
73	L	7	+	+	46	14
74	U	7	−	+	47	54
75	U	7	−	+	48	34
76	V	7	−	+	49	34
77	W	7	−	+	50	34

[a] Different alphabetic character indicates different prefecture where the strain was isolated.
in which the strain isolated.
in which the strain isolated.

Table 4. EHEC O157:H7 isolates from patients used as reference strains

Strains	Origin[a]	*stx1*	*stx2*	PFGE type[b]	Phage type
NIID-2	Hiroshima	+	+	Ⅰa	21
NIID-18	Gifu	+	+	Ⅰb	40
NIID-23	Okayama	+	+	Ⅰc	40
NIID-212	Osaka	+	+	Ⅱa	32
NIID-149	Tokushima	−	+	Ⅲa	ND[c]
NIID-190	Hiroshima	−	+	Ⅲb	ND
NIID-150	Tokushima	−	+	Ⅲc	ND
NIID-875	Shiga	−	+	Ⅲd	ND
NIID-188	Hiroshima	+	+	Ⅲe	ND
NIID-158	Chiba	−	+	Ⅳ	ND
NIID-9	Kanagawa	−	+	Va	26

[a] Name of the prefecture where the strain was isolated.

[b] Classification by NIID of Japan (16).

[c] Not determined.

and Environmental Science (SPIPHE) is one of the reference laboratories for the method in Japan. Dr. Takashi Masuda of SPIPHE carried out the phage typing of the EHEC O157 isolates from cattle.

PFGE

Genomic DNA from each EHEC O157 isolate was prepared by the method of Persing (Mayo Clinic Rochester, MN) published by Rice *et al.* (43) with minor modifications. Isolates were grown in L-broth (Takara Shuzo Co.) for 16-18 h at 37 ℃ with shaking. Fifty μl of the bacterial culture was heated at 100 ℃ for 30 s in a 1.5 ml micro tube, followed by centrifuged at 14,000 g for 3 min. The supernatant was discarded, and the bacterial pellet was suspended in 100 μl 10 mM Tris pH 8.0 containing 100 mM EDTA and 10 mM EGTA (EET). The bacterial suspension was then mixed with equal volume of 1.6 % (W/V) chromosomal grade agarose (Bio Rad Laboratories, Hercules, CA, USA) in EET, placed into disposable agarose plug molds (Bio Rad Laboratories), and cooled for 10 min at 4 ℃. Agarose plugs for each isolate were then placed into sterile 50 ml centrifuge tubes containing 1 ml of EET with 200 μg/ml lysozyme and 0.05 % N-laurlylsarcosine sodium salt (EET-LS) and incubated for 1-3 h in a water bath at 37 ℃. The EET-LS was removed and 1 ml of EET with 1 mg/ml of proteinase K and 1.0 % (W/V) lauryl sulfate sodium (EET-SP) was added to each tube and incubated for 12-18 h in a water bath at 50 ℃. The EET –SP was then removed, the plugs were rinced by 30 min soak in 40 ml of 10 mM Tris pH 8.0 containing 1 mM EDTA (TE), which were repeated four times, and then they were stored at 4 ℃ in TE.

Agarose embedded chromosomal DNA was cleaved with an endonuclease (either *Xba* I or *Bln* I : Takara Shuzo Co.) following the manufacturer's directions. PFGE was performed in a 1% pulsed field certified agarose (Bio Rad Laboratories) using CHEF DR Ⅲ apparatus (Bio Rad Laboratories) in 0.5×TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA) at 10℃ at 200 V. The pulse time for *Xba* I was increased from 2 to 50 s for 20 h. For separation of bands less than 100 kb, a constant switching time 4 s was applied for 16 h. Pulse time for *Bln* I was increased from 2 to 50 s for 20 h. Lambda ladders (Bio Rad Laboratories) were used as size markers.

Comparison of the discriminatory ability of the epidemiological markers

The discriminatory power of the epidemiological markers, i.e., toxin profile, phage type, and PFGE, was compared each other by a numerical index of discrimination, namely Simpson's index of diversity (Simpson's D) (15). This index is based on the probability that two unrelated strains sampled from the test population will be placed into different typing groups, and given by the following equation:

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{s} n_j (n_j - 1)$$

where N is the total number of strains in the sample population, s is the total number of types described, and n_j is the number of strains belonging to the jth type.

Cluster analysis

Digestion profiles of bovine and human isolates by *Xba* I were compared with each other by using the Dice similarity coefficient and Diversity Database Software (PDI, Huntington Station, NY). Cluster analysis was done using the unweighted pair group method using arithmetic average (UPGMA) (maximum tolerance, 2.0%), and a dendrogram was prepared.

Results

Subtyping of EHEC O157 isolates from cattle in Japan

Fig. 1 shows the examples of toxin profiles of the EHEC O157 isolates. Among the 77 EHEC O157 isolates from cattle, 75 isolates (97.4%) had at least one of *stx1* and *stx2* genes, and 28 isolates (36.4%) had genes for both *stx1* and *stx2*. Forty-three isolates (55.8%) possessed the gene for *stx2* only and 4 isolates (5.2%) possessed the gene for *stx1* only. The remaining 2 isolates (2.6%) had neither *stx* gene.

Fifteen phage types were observed among the bovine isolates: 1, 2, 4, 8, 14, 21, 31, 32, 34, 39, 40, 54, 70, 71, and 74 (Fig.2 and Table 3). Three phage types, 21 (16 isolates), 54 (16 isolates) and 34 (11 isolates), were dominant, and the other types were represented by six or fewer isolates.

On the other hand, 50 *Xba* I -PFGE profiles were observed among 77 bovine isolates (Table 3). Fig. 3 shows the representative PFGE profiles of the isolates. The Simpson's D for *Xba* I -PFGE was the highest value of 0.987, while that for toxin profile and phage type were 0.560 and 0.884, respectively (Table 5). This index indicates that if two strains were sampled randomly from the population, then on 98.7 % of occasions they would fall into different PFGE types.

Cluster analysis

Cluster analysis was done by using 50 *Xba* I digestion profiles from bovine isolates and 11 *Xba* I digestion profiles from human isolates. Nine clusters were observed at a similarity coefficient of 0.58 (Fig. 2). Samples with similarity coefficient of 0.88 or more were closely related. The number of band of sizes different from that in each other was less than three. On the other hand, the number of band of sizes different from that in each other was six or more in samples with similarity coefficient of less than 0.80. In samples with the similarity coefficient of less than 0.40, the number of band of sizes different from that in each other was more than 15. Human isolates were distributed among cluster B, F, H, and I (Fig. 2). All four profiles of cluster D and 15 of 18 profiles of cluster H were observed among isolates having both *stx1* and *stx2* genes. On the other hand, all the profiles of cluster A, C, E, F, G and I were observed among isolates having the *stx2* gene only or neither *stx* gene (Fig. 2). Dominant phage

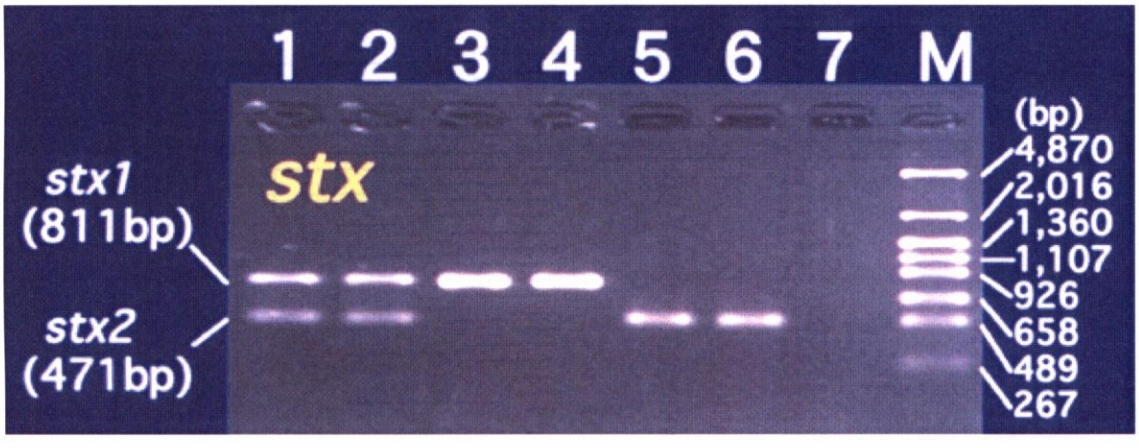

Fig. 1. Toxin profiles from EHEC O157:H7 isolates from cattle in Japan. Lane 1 and 2 are isolates having genes for both *stx1* and *stx2*. Lane 3 and 4 are isolates having gene for *stx1* only. Lane 5 and 6 are isolates having gene for *stx2* only. Lane 7 is isolate having neither genes. M indicates a pHY marker as a size marker.

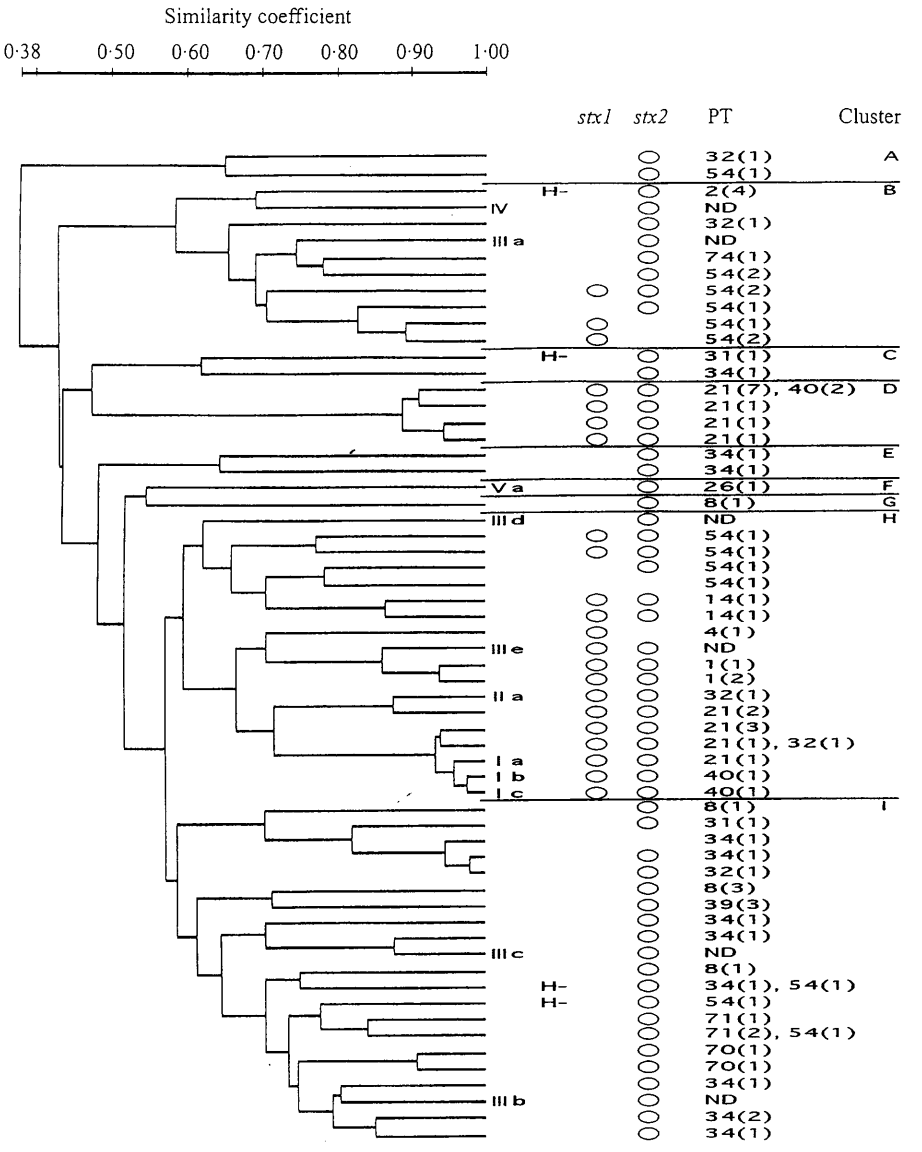

Fig. 2. Comparison of bovine and human EHEC O157:H7 isolates based on the cluster analysis of *Xba* I digestion profiles with the toxin profile and phage type. Roman numerals indicate the classification of the PFGE profiles of human isolates by the NIID of Japan (16). H- indicates EHEC O157:H- isolates. PT is the abbreviation for phage type. The numbers in the parenthesis indicate the number of isolates. Nine clusters were observed at a similarity coeffcient of 0.58 and are designated A to I.

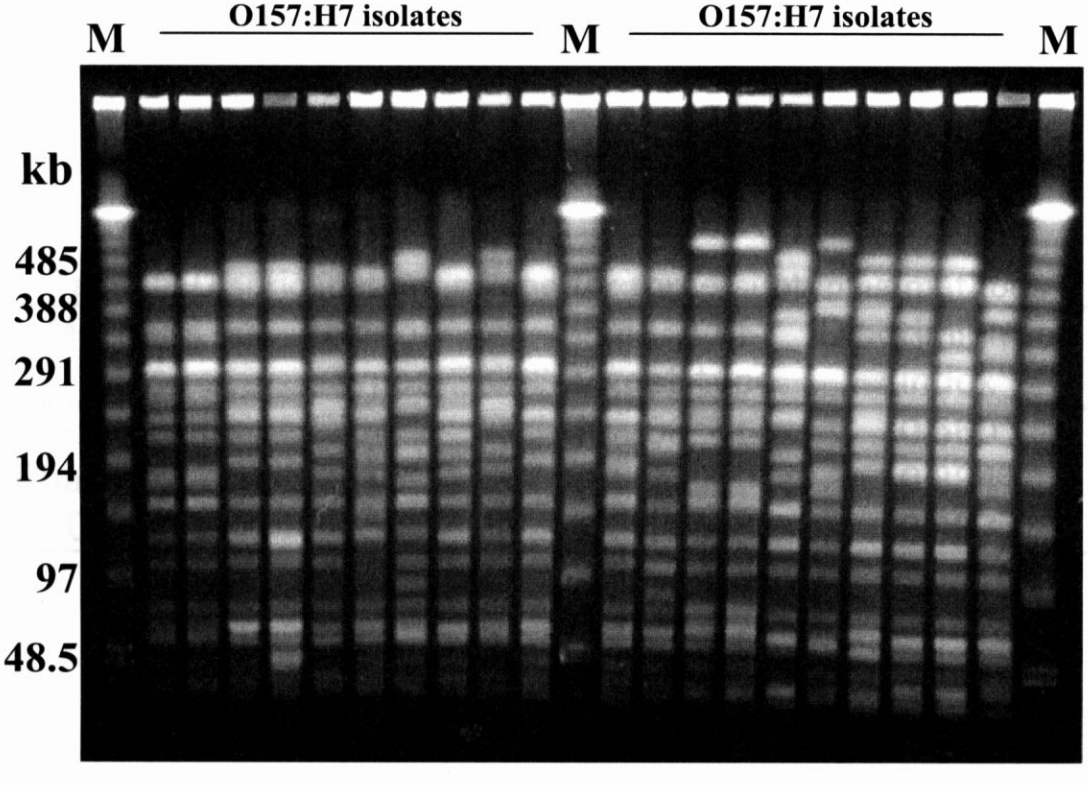

Fig. 3. Representative PFGE profiles of EHEC O157:H7 isolates from cattle in Japan. M indicates a Lambda ladder as a size marker.

Table 5. Discrimination indices for three subtyping methods

Methods	No. of types	Size (%) of largest type	Discrimination index
Toxin profile	4	56	0.560
Phage type	15	21	0.884
PFGE type	50	12	0.987

types 21, 54 and 34 were each distributed among two or more different clusters (Fig. 2). Isolates with indistinguishable PFGE profiles were of multiple phage types (Fig. 2).

Comparison of bovine and human isolates

Seven EHEC O157:H7 isolates from cattle had similar *Xba* I PFGE profiles to human isolates : 5 strains (Nos. 8, 9, 53, 54, and 55 in Table 3) from 2 prefectures had types I a and I c profiles and 2 strains (Nos. 30 and 34 in Table 3) from 1 prefecture had a type II a profile (Fig. 4a and a', Table 3). Four of these 7 bovine isolates (Nos. 8, 9, 54, and 55 in Table 3) with types I a and I c profiles were indistinguishable from human isolates by *Bln* I digestion. (Fig. 4 b). One of the bovine isolates (No. 9 isolate in Table 3) was also indistinguishable from human isolates by phage typing (Table 6).

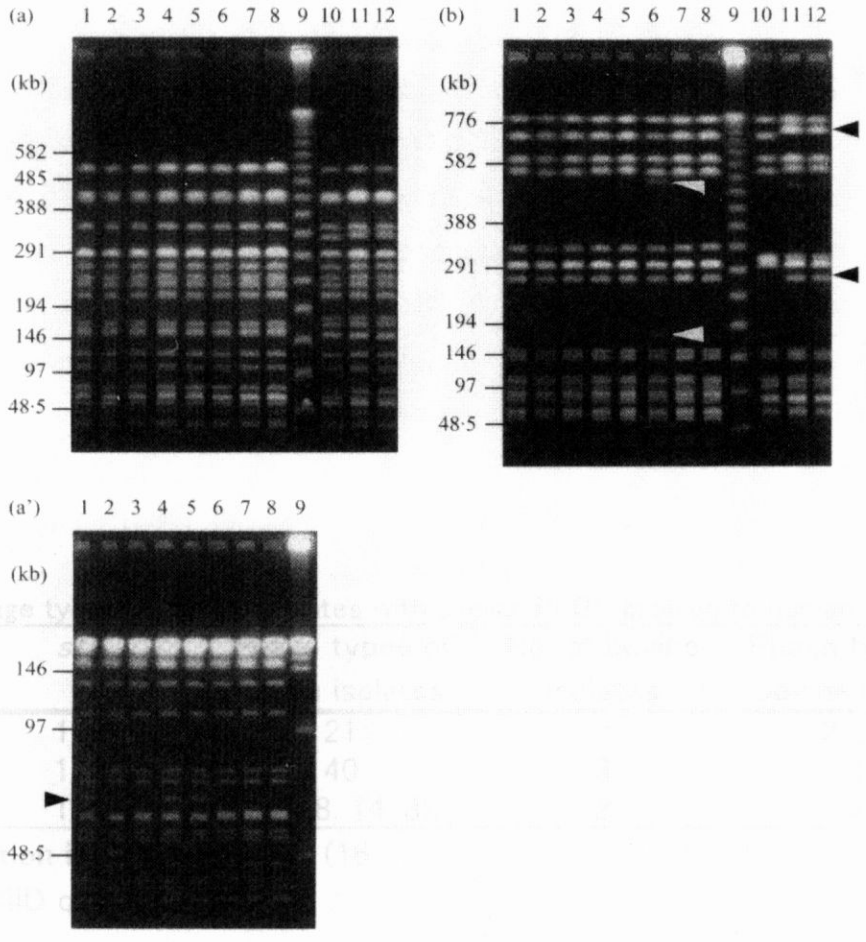

Fig. 4. Bovine isolates with *Xba* I digestion profiles similar to those of human isolates (a, a') and discrimination of the same isolates as described in panel (a) by *Bln* I digestion (b). The panels (a) and (b) are the profiles obtained by using the PFGE parameter 2 to 50 s for 20 h to separate all the fragments generated while the panel (a') is the profile obtained by using the parameter 4 s for 16 h to separate fragments less than 100 kb in size. Lanes 1, 2, 3, and 10 are the human isolates belonging to PFGE types I a (NIID-2), I b (NIID-18), I c (NIID-23), and II a (NIID-212) shown in Table 4, respectively. Lanes 4, 5, 6, 7, 8, 11, and 12 are the bovine isolates and correspond to Nos. 8, 9, 53, 54, 55, 30, and 34, respectively, in Table 3. Lane 9 is a Lambda ladder as a size marker. Lanes 4 and 5 are isolates from the F prefecture and have a 75 kb band (arrowhead on a') specific for type I a. Lanes 6-8 are from the M prefecture and have the same PFGE pattern for bands less than 100 kb as a type I c human isolate. Lanes 11 and 12 are the bovine isolates with the same PFGE patterns as type II a human isolate. Of these bovine isolates, bovine type II a isolates (Lanes 11 and 12) was clearly discriminated from the human type II a isolate (Lane 10) by difference in size of the second largest band (upper black arrowhead on b) and by the presence of a 280 kb band (lower black arrowhead on b) by *Bln* I digestion. One of the bovine type I c isolates (Lane 6) was also discriminated from human type I c isolate by the presence of a 500 and a 180 kb band (white arrowheads on b). The remaining type I isolate from cattle (Lanes 4, 5, 7, and 8) could not be discriminated from human isolates (Lanes 1-3) by *Bln* I digestion.

Table 6. Phage types of bovine isolates with similar PFGE profiles to human isolates

PFGE type[a]	*stx*	Phage types of human isolates[b]	No. of bovine isolates	Phage types of bovine isolates
Ⅰa	1, 2	21	2	21, 32
Ⅰc	1, 2	40	3	21
Ⅱa	1, 2	1, 4, 8, 14, 32	2	21

[a] Classification by NIID of Japan (16).

[b] Data by NIID of Japan (17).

Discussion

In this investigation, a total of 77 EHEC O157:H7 (H-) isolates from cattle in Japan were investigated by molecular biological methods. Most of these isolates (43 isolates) possessed the *stx2* gene, but not *stx1*. Fifteen phage types and fifty PFGE profiles were observed. These results indicate that EHEC O157:H7 strains with various genetic subtypes have already spread to cattle in Japan.

PFGE is widely used as a molecular subtyping method of EHEC O157:H7 because of its high discriminatory power and good reproducibility (3, 6, 16, 26, 29, 31). In this study, the Simpson's D for PFGE was 0.987. This strongly suggests that PFGE has sufficient discriminatory ability to subtype bovine EHEC O157:H7 isolates in Japan and can be used as a powerful epidemiologic tool.

In general, the discriminatory power of phage typing is lower than that of subtyping by PFGE. Krause *et al.* (26) reported that the Simpson's D was 0.786 for phage types and 0.987 for PFGE types of *E. coli* O157:H7. In this study, the Simpson's D for phage types was a relatively high value of 0.884. The results of phage typing and PFGE subtyping were discordant in that each phage type was distributed throughout several PFGE defined clusters. These results suggest that a combined use of phage typing and PFGE would provide a more detailed classification of isolates.

Though PFGE was the best method for subtyping of EHEC O157:H7 isolates from the aspect of discriminatory power, the procedure is generally complicated and time-consuming. For example, it needs 3 to 4 days to get the typing results by using the PFGE protocol standardized by NIID of Japan (48). Gautom (11) reported a rapid PFGE protocol for typing of EHEC O157:H7 and other gram-negative organisms in 1 day. PFGE is now recognized as a gold standard for subtyping of EHEC O157:H7.

Four bovine isolates were indistinguishable from human isolates by PFGE using two kinds of endonucleases. One of the four isolates was also indistinguishable from human isolates by phage typing. These results strongly suggest that the bovine isolate is the same clone as the human isolates although there was no epidemiologic information concerning connection between these bovine and human outbreaks. 12 phage types that we found among bovine isolates were also observed in human EHEC O157:H7 isolates in Japan (17). These data indicate that cattle should be considered as

a possible source of human EHEC O157:H7 infections in Japan as well as North America and Europe. Effective on-farm control measures of this pathogen are needed to prevent human EHEC O157:H7 infections in Japan.

Summary

A total of 77 EHEC O157:H7(H-) isolates from cattle in Japan were investigated by molecular biological methods. Most of these isolates (43 isolates) possessed the *stx2* gene, but not *stx1*. Fifteen phage types and fifty PFGE profiles were observed. These results indicate that EHEC O157:H7 strains with various genetic subtypes have already spread to cattle in Japan.

In this study, the Simpson's D for PFGE types and phage types were 0.987 and 0.884, respectively. This suggests that PFGE has sufficient discriminatory ability to subtype bovine EHEC O157:H7 isolates in Japan and can be used as a powerful epidemiologic tool. The results of phage typing and PFGE typing were discordant in that each phage type was distributed throughout several PFGE defined clusters. These results suggest that a combined use of phage typing and PFGE would provide a more detailed classification of EHEC O157:H7 isolates.

One isolate was indistinguishable from the human outbreak strain by these methods. This indicates that cattle must be considered as a possible source of human EHEC O157:H7 infections in Japan.

Chapter Ⅱ

Fecal shedding patterns and shift of genetic subtypes of EHEC O157:H7 in naturally infected cattle

Introduction

In the last chapter, the results of an evaluation of epidemiological markers, i.e., toxin profile, phage type, and PFGE, as a method of subtyping EHEC O157:H7 isolates from cattle in Japan were addressed. PFGE has a sufficient discriminatory ability to subtype bovine EHEC O157:H7 isolates in Japan and can be used as a powerful epidemiologic tool. Moreover, by these methods, one isolate was indistinguishable from the human outbreak strain. This indicates that cattle must be considered a possible source of human EHEC O157:H7 infection in Japan.

According to the results of on-farm survey conducted by the Ministry of Agriculture, Forestry, and Fisheries of Japan in 1996, EHEC O157:H7 was isolated from 0.62% of cattle fecal samples (45). However the epidemiology of this agent on cattle farms is still unclear in Japan.

In order to clarify the ecology of EHEC O157:H7 on farms from the aspect of a possible source of human EHEC O157:H7 infection, the duration and magnitude of fecal shedding of EHEC O157:H7 by naturally infected cattle on one farm were investigated. Moreover, the genotypes of the EHEC O157:H7 isolates obtained by sequential fecal cultures were studied by using PFGE.

Materials and Methods

Determination of EHEC O157:H7 shedding cattle on a farm

Fecal samples were collected from 670 cattle of farm A in Japan on September 20 or October 3 in 1996. Direct culture was performed by using MacConkey sorbitol agar (Difco, Detroit, MI) plates with 50 ng/ml cefixime (Dynal, Oslo, Norway) and 2.5 μ g/ml tellurite (Dynal) (SMAC-CT) (50). Sorbitol non-fermenting colonies were streaked onto a Tripticase soy agar (Becton Dickinson, Cockeysville, MD, USA) plates and incubated at 37℃ for 18 h. The isolates were tested for agglutination by *E. coli* O157 and H7 antisera (Denka Seiken Co., Tokyo, Japan) according to the manufacturer's instructions. Agglutination-positive isolates were confirmed as *E. coli* by biochemical tests with the API 20E (bio Mérieux sa, Etoile, France). Cattle of which EHEC O157:H7 was isolated from their feces at this point in time were considered as EHEC O157:H7 shedders and used for the following investigation.

Sequential fecal culture

Sequential fecal culture of EHEC O157:H7 shedder was performed by the method of Chapman *et al.* (7) with minor modification. Briefly, 1 g of feces were added to 10 ml of saline in capped tubes, and serially diluted with saline for direct plating. SMAC-CT were inoculated with 0.1 ml of the diluted fecal samples, and incubated at 37 ℃ for 18 hours. For enrichment, 1 g of feces was inoculated to 10 ml of broth enrichment media (mEC; Kyokuto Pharmaceutical Co., Tokyo, Japan) supplemented with 20 μ g/ml novobiocin (Sigma, St Louis, MO, USA) and incubated at 42 ℃ for 20 hours. After being concentrated by immunomagnetic separation (IMS) using immunobeads (Dynabeads [anti-*E. coli* O157:H7]; Dynal), the beads were spread on SMAC-CT and incubated at 37 ℃ for 18 hours. Up to 12 sorbitol non-fermenting colonies were selected from each SMAC-CT plate and identified as EHEC O157:H7 by the methods described above. The most probable number (MPN) of EHEC O157:H7 per 1 g of feces was determined by using the enrichment broth media (24).

Characterization of the isolates

All the EHEC O157:H7 isolates recovered from cattle were analyzed by using following

methods. For PFGE analysis, genomic DNA of each isolate was prepared by the procedure explained in Chapter Ⅰ. Agarose embedded chromosomal DNA was cleaved with an endonuclease (*Xba* Ⅰ, *Bln* Ⅰ, and *Spe* Ⅰ: Takara Shuzo Co.) following the manufacturer's directions. PFGE was performed in a 1% pulsed field certified agarose (Bio Rad Laboratories) using CHEF DRⅢ apparatus (Bio Rad Laboratories) in 0.5×TBE buffer at 10℃ at 200 V. The pulse time for *Xba* I was increased from 2 to 50 s for 20 h. For separation of bands less than 100 kb, a constant switching time of 4 s was applied for 16 h. Pulse time for *Bln* I was increased from 2 to 50 s for 20 h. And pulse time for *Spe* I was increased from 10 to 20 s for 22h. Lambda ladders (Bio Rad Laboratories) were used as size markers. The numbers of band of sizes different from the inoculated isolates (Band differences) were determined by enumerating the loss and addition of bands. PCR for detection of the *stx* genes was performed by the procedure described in Chapter Ⅰ.

Results

Detection of EHEC O157:H7 from fecal samples

As shown in Table 7, EHEC O157:H7 isolates were detected from seven of 670 cattle of farm A in Japan on September 20 or October 3 in 1996. All animals were clinically normal. These seven cattle were used for the following investigation. Sequential fecal samples of these cattle were collected once every week from October 30 to December 4 in 1996 (a total of six times). As shown in Table 8, EHEC O157:H7 was isolated more than once in four of the seven cattle. Three other cattle were culture negative through this period. The MPNs of EHEC O157:H7 per 1 g of feces were less than 50.

Molecular genetic characteristics of the isolates

Thirty-nine EHEC O157 isolates were obtained from nine positive fecal samples collected from October 30 to November 20. Together with seven isolates from fecal samples collected on September 20 and October 3, a total of 46 *E. coli* O157 isolates were analyzed.

All isolates were subtyped using PFGE patterns. Among the 46 EHEC O157:H7 isolates, 4, 6 and 4 PFGE patterns were observed by *Xba* I , *Bln* I and *Spe* I digestion, respectively (Table 9). The author named the dominant PFGE pattern as type1 in each restriction endonuclease. A total of nine genetic subtypes (I ～IX) were observed by a combination of these PFGE types (Table 9). The number of fragment differences from type 1 was less than four in each enzyme except for the isolate 6-0 (Fig. 5 and Table 9).

While most of the isolates possessed the gene for *stx2* only, neither gene was detected from the 11 isolates belonging to genetic subtypes III, IV, V and VI from cattle No.3 (Table 9).

The shift of genetic subtypes of the isolates

In four of the seven cattle, EHEC O157:H7 was isolated more than twice from the feces collected from September 20 to November 20. The shift of PFGE pattern was observed in three of the four cattle. Especially, after November 6, changes in the genetic group of the isolates were observed every week in cattle No. 3 (Table 9).

Table 7. EHEC O157:H7 shedding cattle on one farm

No.	Breed	Month old	Gender	Raising place
1	Japanese Black	5	Male	A
2	Japanese Black	5	Female	B
3	Holstein	6	Female	B
4	Holstein	5	Female	B
5	Holstein	3	Female	C
6	Japanese Black	18	Female	D
7	Japanese Black	13	Female	D

Table 8. Isolation of EHEC O157:H7 from fecal samples and determination of the MPNs

Cattle No.	Sampling date							
	Sep 20	Oct 3	Oct 30	Nov 3	Nov 13	Nov 20	Nov 27	Dec 4
1	NT	+a	−b	−	−	−	−	−
2	NT	+	−	−	−	−	−	−
3	+	NT	+ (43)c	+ (23)	+ (9)	+ (4)	+ (<3)	−
4	+	NT	−	−	−	+ (<3)	−	−
5	+	NT	−	−	−	−	−	−
6	NT	+	−	−	−	+ (9)	−	−
7	NT	+	−	+ (9)	−	+ (4)	−	−

a EHEC O157:H7 positive.
b EHEC O157:H7 negative.
c MPN of EHEC O157:H7 per 1 g of feces.

Table 9. Genotypic characteristics of EHEC O157:H7 isolates from cattle on one farm

Cattle No.	Isolate No.	Date of isolation	*stx2*	PFGE type			Genetic subtype
				Xba I	*Bln* I	*Spe* I	
1	1-0	3 Oct 1992	+	1	1	1	I
2	2-0	3 Oct 1992	+	1	1	1	I
3	3-0	20 Sep 1992	+	1	1	1	I
	3-1-1	30 Oct 1992	+	1	2(1)[a]	1	II
	3-1-2	30 Oct 1992	+	1	1	1	I
	3-1-3	30 Oct 1992	+	1	1	1	I
	3-1-4	30 Oct 1992	+	1	1	1	I
	3-1-5	30 Oct 1992	+	1	1	1	I
	3-1-6	30 Oct 1992	+	1	1	1	I
	3-1-7	30 Oct 1992	+	1	1	1	I
	3-1-8	30 Oct 1992	+	1	1	1	I
	3-1-9	30 Oct 1992	+	1	1	1	I
	3-1-10	30 Oct 1992	+	1	1	1	I
	3-1-11	30 Oct 1992	+	1	1	1	I
	3-2-1	6 Nov 1992	+	1	1	1	I
	3-2-2	6 Nov 1992	+	1	1	1	I
	3-3-1	13 Nov 1992	−	1	3(2)	2(2)	III
	3-3-2	13 Nov 1992	−	1	3	2	III
	3-3-3	13 Nov 1992	−	1	4(3)	2	IV
	3-3-4	13 Nov 1992	−	1	3	2	III
	3-3-5	13 Nov 1992	−	1	3	2	III
	3-3-6	13 Nov 1992	−	1	3	2	III
	3-3-7	13 Nov 1992	−	1	3	2	III
	3-4-1	20 Nov 1992	+	1	1	1	I
	3-4-2	20 Nov 1992	+	1	1	1	I
	3-4-3	20 Nov 1992	+	1	1	1	I
	3-4-4	20 Nov 1992	+	1	1	1	I
	3-5-1	27 Nov 1992	−	2(2)	3	2	V
	3-5-2	27 Nov 1992	−	2	5(2)	2	VI
	3-5-3	27 Nov 1992	−	2	5	2	VI
	3-5-4	27 Nov 1992	−	2	5	2	VI
4	4-0	20 Sep 1992	+	1	1	1	I
	4-4-1	20 Nov 1992	+	1	1	1	I
5	5-0	20 Sep 1992	+	1	1	1	I
6	6-0	3 Oct 1992	+	3(7)	3	3(5)	VII
	6-4-1	20 Nov 1992	+	1	1	1	I
	6-4-2	20 Nov 1992	+	1	1	1	I
	6-4-3	20 Nov 1992	+	1	1	1	I
	6-4-4	20 Nov 1992	+	1	1	1	I
7	7-0	3 Oct 1992	+	4(3)	1	4(2)	VIII
	7-2-1	6 Nov 1992	+	1	6(3)	1	IX
	7-2-2	6 Nov 1992	+	1	6	1	IX
	7-4-1	20 Nov 1992	+	1	1	1	I
	7-4-2	20 Nov 1992	+	1	1	1	I
	7-4-3	20 Nov 1992	+	1	1	1	I
	7-4-4	20 Nov 1992	+	1	1	1	I

[a] Number of band of sizes different from that in type 1.

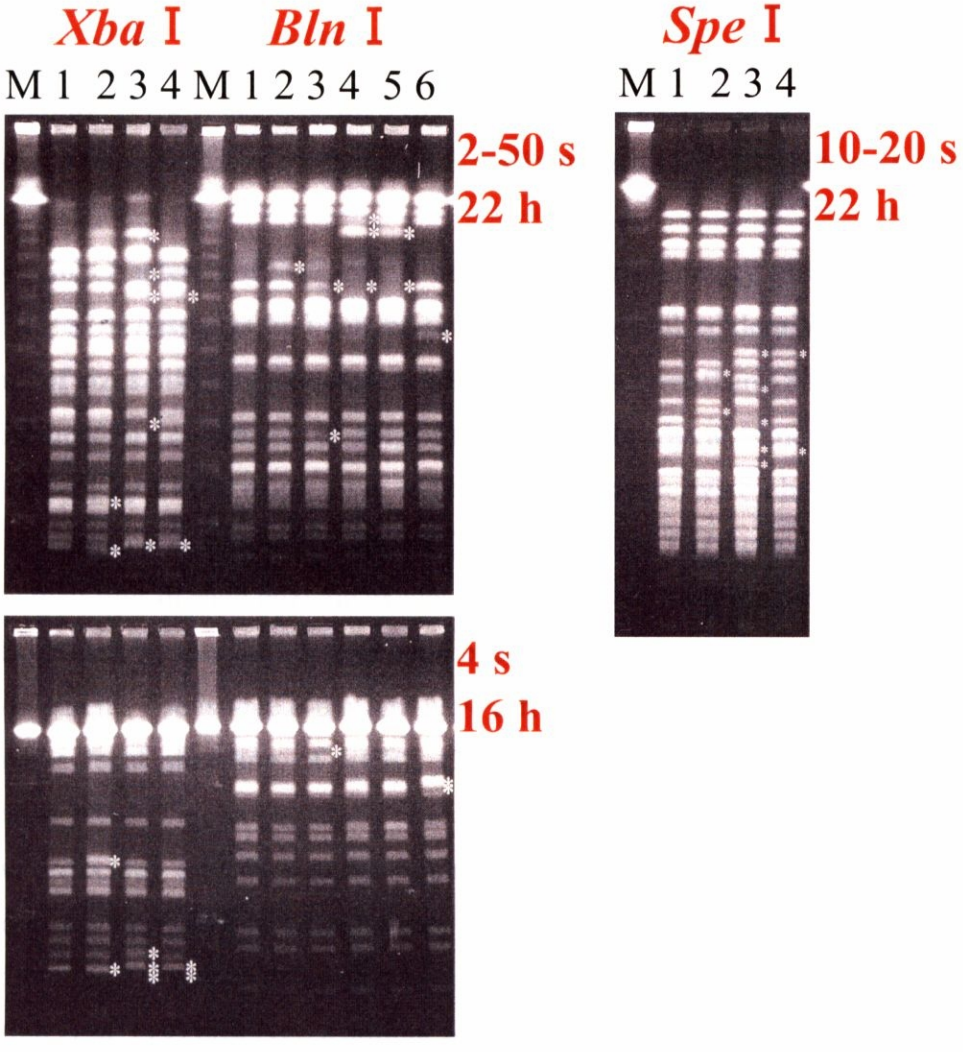

Fig. 5. Variation of PFGE profiles of EHEC O157:H7 isolates from cattle on one farm. The endonucleases and PFGE conditions used were indicated red characters. The numbers on each lane correspond to the PFGE types shown in Table 9. M indicates a Lambda ladder as a size marker. The asterisks indicate the bands of sizes different from those in type 1 (Lane 1).

Discussion

Four of the seven cattle shed EHEC O157:H7 intermittently in this investigation. Though the correct duration of fecal shedding was not clear, the magnitude of that was small. Moreover, all the EHEC O157:H7 shedder was clinically normal through the investigation period. As Hancock *et al.* (13) pointed out, EHEC O157:H7 appears to behave similarly to the most of nonpathogenic *E. coli* strains, rather than being a pathogen in cattle.

As described in Chapter I , PFGE has a sufficient discriminatory ability to subtype bovine EHEC O157:H7 isolates and can be used as a powerful epidemiologic tool. The number of band of sizes different from that in each other was more than 15 in samples with similarity coefficient of less than 0.40 (Fig. 1). On the other hand, nine closely related subtypes determined by PFGE using three kind of endonuclease were observed in the 46 EHEC O157:H7 isolates in this investigation. The number of fragment differences from type 1 was less than four in each enzyme except the isolate 6-0. As these differences can be theoretically explained by less than two genetic events (47), these isolates may be derived from a common parent. The isolate 6-0 might have different origin than the other isolates.

The continuous fecal shedding of EHEC O157:H7 for more than two months was observed in cattle No.3. The author also observed a shift of PFGE profile in the isolates from this cow. Especially after November 6, changes in the genetic group of the isolates were observed every week. While most isolates possessed the *stx2* gene only, neither gene was detected from the 11 isolates belonging to genetic subtypes Ⅲ, Ⅳ, Ⅴ and Ⅵ from cow No.3. Karch *et al.* (19) observed a similar phenomenan in human patients. That is, the shift of PFGE profile and the loss of *stx2* gene were observed among EHEC O157:H7 isolates from three of seven long-term human shedders. They suggested that the loss of *stx2* gene could be resulted from the genetic changes detectable by PFGE. It is not clear that the shift of PFGE pattern in the isolates from cattle No.3 was represented by a reinfection from the environment, a double infection, or a result of genetic changes and clonal replacement on the dominant genetic subtype within the cow. Since all but one isolate used in this study, however, were highly related, we assumed that a single strain that colonized cattle on this farm could have mutated slightly during either carriage by cattle, or in the farm environment.

The shift of PFGE pattern was also observed in two other cattle. The fact that the shift of genetic subtype was observed in three of the four cattle from which EHEC O157:H7 were isolated more than twice, including the examination of September 20 or October 3, is important for investigators who wish to make inferences regarding genetic relatedness of EHEC O157:H7 isolates using PFGE patterns.

Faith *et al.* (9) observed up to three distinguishable, but closely related PFGE patterns among multiple isolates from the same cow. Keene *et al.* (21) also observed seven distinguishable PFGE patterns in three homology groups which differed by less than three bands among human and dairy herd EHEC O157:H7 isolates in a prolonged outbreak caused by raw milk. They suggested that outbreaks are not necessarily marked by a single pattern. The observations in this chapter corroborate this opinion. Similarity, rather than exact matching, arguably gives a better understanding of the outbreak of EHEC O157:H7.

Summary

Seven EHEC O157:H7 shedding cattle were determined in one farm. Sequential fecal samples of these cattle were collected and cultured every week for about two months. EHEC O157:H7 was isolated more than once in four of the seven cattle. Three other cattle were culture negative through this period. The MPNs of EHEC O157:H7 per 1 g of feces were less than 50.

A total of 46 EHEC O157:H7 isolates were obtained from sequential fecal samples from the seven cattle. Nine closely related genetic subtypes, determined by PFGE types using three kinds of restriction endonuclease were observed among the isolates. Moreover, the shift of genetic subtypes was observed in three of the four cattle from which EHEC O157:H7 were isolated more than twice. Distinguishable, but closely related genetic subtypes can be isolated from one farm, or from one cow, should be considered when undertaking an epidemiological survey. Similarity, rather than exact matching, arguably gives a better understanding of the outbreak of EHEC O157:H7.

Chapter Ⅲ

Fecal shedding patterns and shift of genetic subtypes of EHEC O157:H7 in experimentally infected cattle

Introduction

In the former chapter, the duration and magnitude of fecal shedding of EHEC O157:H7 by naturally infected cattle on one farm were investigated in order to clarify the ecology of EHEC O157:H7 on farms. Sequential fecal samples from seven EHEC O157:H7 shedding cattle on one farm were collected and cultured every week for about two months. As a result, EHEC O157:H7 was isolated more than once in four of the seven cattle. The continuous fecal shedding of EHEC O157:H7 for more than two months was observed in cattle No.3. We also observed a shift of PFGE profile in the isolates from this cow. Especially after November 6, changes in the genetic group of the isolates were observed every week.

Karch *et al.* (19) observed a similar phenomenon in human patients. That is, the shift of PFGE profile was observed among EHEC O157:H7 isolates from three of seven long-term human shedders. They referred the genetic changes and clonal replacement of this organism observed in human patients infected with EHEC O157:H7 to clonal turnover. It is not clear that the shift of PFGE pattern in the isolates from cattle No.3 was represented by a reinfection from the environment, a double infection, or a result of genetic changes and clonal replacement on the dominant genetic subtype within the cow. The purpose of this chapter is to study whether clonal turnover takes place within experimentally infected cattle.

Materials and Methods

Experimental animals and inoculation

Three 8-week-old Holstein steers were housed individually in climate-controlled BSL-2 containment barns in accordance with the guidelines of animal experimentation defined by National Institute of Animal Health of Japan. Pens had individual floor drains and were cleaned twice daily with water and disinfectant. The calves were fed milk replacer without antibiotics in the morning and afternoon. All animals were healthy and culture negative for EHEC O157 strains as determined by a technique described in Chapter II prior to inoculation. EHEC O157:H7 strain 3-0 (see Chapter II), which was isolated from cattle in Japan in 1996, was used for inoculation. This strain harbors a single 90 kb-sized plasmid, and the genes for *stx2* and *eaeA*, but not *stx1* was detectable by PCR. Two of three calves (Nos. 1 and 2) were inoculated via a stomach tube with 10^9 CFU log-phase culture of EHEC O157:H7 strain 3-0 which had been grown in Tripticase soy broth (Becton Dickinson). The remaining calf (No. 3) was used as an uninoculated control. Fecal samples of these calves were obtained once daily for 63 days by digital rectal retrieval. Body temperature, condition of feces, and other clinical status of the three calves were checked once daily throughout the study.

Isolation of EHEC O157:H7

Fecal culture was performed by the procedure explained in Chapter II. Briefly, 1 g of feces were added to 10 ml of saline in capped tubes, and serially diluted with saline for direct plating. SMAC-CT were inoculated with 0.1 ml of the diluted fecal samples, and incubated at 37 ℃ for 18 hours. For enrichment, 1 g of feces were inoculated to 10 ml of broth enrichment media (mEC; Kyokuto Pharmaceutical Co.) and incubated at 42 ℃ for 20 hours. After concentration by IMS, the beads were spread on SMAC-CT and incubated at 37 ℃ for 18 hours. Up to 10 sorbitol non-fermenting colonies were selected from each SMAC-CT plate and identified as EHEC O157:H7 by the methods described in Chapter II. The MPN of EHEC O157:H7 per 1 g of feces was determined by using enrichment broth media (24).

Analysis of the isolates

All the EHEC O157:H7 isolates recovered from calves were analyzed by using

following methods. Ten colonies of EHEC O157:H7 strain 3-0 obtained by subcultivation on SMAC-CT were used as positive controls for each analysis.

For PFGE analysis, genomic DNA of each isolate was prepared by the procedure described in Chapter I . Agarose embedded chromosomal DNA was cleaved with an endonuclease (*Xba* I : Takara Shuzo Co.) following the manufacturer's direction. PFGE was performed in 1% agarose gels using a CHEF DRIII apparatus (Bio Rad Laboratories) in 0.5× TBE at 10 ℃ at 200 V. The pulse time was increased from 15 to 40 s over 24 h. To fractionate and visualize less than 100 kb bands, a pulse time of 4 s over 20 h was applied. Lambda ladders (Bio Rad) were used as size markers. The numbers of band of sizes different from the inoculated isolates (band differences) were determined by enumerating the loss and addition of bands.

Plasmid DNA was obtained by the method of Birnboim and Doly (5) with minor modifications. Briefly, isolates were grown in L- broth (Takara Shuzo Co.) at 37℃ for 16-18 h. One ml of the culture was pelleted by centrifugation (15,000 rpm, 30 s). The cell pellet was thoroughly suspended in 100 μl of Solution 1 [50 % glucose 1.8 ml, 0.5 M EDTA (pH 8.0) 2.0 ml, 1 M Tris-HCl (pH 8.0) 2.5 ml, ultrapure water 53.7 ml]. The cells were lysed by adding 200 μl of Solution 2 (0.2 N NaOH, 1 % SDS). The solution was added 150 μl of Solution 3 (phenol: chloroform: isoamyl alcohol=25:24:1) and emulsified by shaking. Then the solution was centrifuged at 15,000 rpm for 7 min. 10 μl of the aqueous phases were applied to 0.5 % agarose gels and electrophoresis was performed in TBE buffer.

PCR was used for detection of the *stx2* and *eaeA* genes (22, 35). Briefly, a primer pair V3 (5' TTCGGTATCCTATTCCCG) and V4 (5' TCTCTGGTCATTGTATTA) was used to amplify a 471 bp DNA fragment from the *stx2* gene. A primer pair eaeK1 (5'-GCTTAGTGCTGGTTTAGGAT) and eaeK4 (5'-TCGCCGTTCAGAGATCGC) was used to amplify a 489 bp DNA fragment from the *eaeA* gene. Bacterial DNA was amplified using a TaKaRa PCR Thermal Cycler TP2000 (Takara Shuzo Co.), and electrophoresis was performed in 2% agarose gels and TBE buffer.

Results

Animal Health

All animals were not febrile and had normal feces throughout the study even after the inoculation of 10^9 CFU of the EHEC O157:H7 strain 3-0. All calves also maintained a normal appetite and remained alert and responsive.

Duration and magnitude of fecal shedding of EHEC O157:H7

EHEC O157:H7 inoculated calves (Nos. 1 and 2) were culture positive for the organism 24 h after inoculation. Calf No.1 shed from 10^5 to 10^7 CFU of EHEC O157:H7 until 7 days post inoculation. Subsequently, the measured level of EHEC O157:H7 present in the feces decreased and remained between 4 and 10^2 CFU until 49 days post inoculation (Fig. 6A). EHEC O157:H7 was detected in the feces of Calf No. 2 shed from one to five days post inoculation at levels between 10^5 and 10^7 CFU. The level of fecal EHEC O157:H7 from days 6 to 50 did not exceed 10^4 CFU (Fig. 6B). All the EHEC O157:H7 isolates obtained on and after 9 days post inoculation were isolated from enrichment culture only. EHEC O157:H7 was not recovered after 50 days post inoculation from the inoculated calves even by using enrichment culture. The uninoculated control calf No.3 was culture negative through the experimental period.

Isolation and analysis of the isolates

Two hundred and fifteen isolates and 186 isolates of EHEC O157:H7 were recovered from calf Nos. 1 and 2, respectively. A total of 401 isolates were analyzed by PFGE, plasmid profile, and PCR for *stx2* and *eaeA* genes.

Among 215 isolates from calf No. 1, 109 isolates had different PFGE profiles from that of the inoculated strain. Thirty-eight of 186 isolates from calf No. 2 also had different PFGE profiles (Table 10). The author calls these isolates "mutated isolates". Seventeen and 10 different PFGE subtypes were observed among the 109 mutated isolates from calf No. 1 and the 38 mutated isolates from calf No. 2, respectively (Table 10). The numbers of fragment differences from the inoculated strain were from one to six. A common PFGE profile was not observed among the mutated isolates between the two inoculated calves.

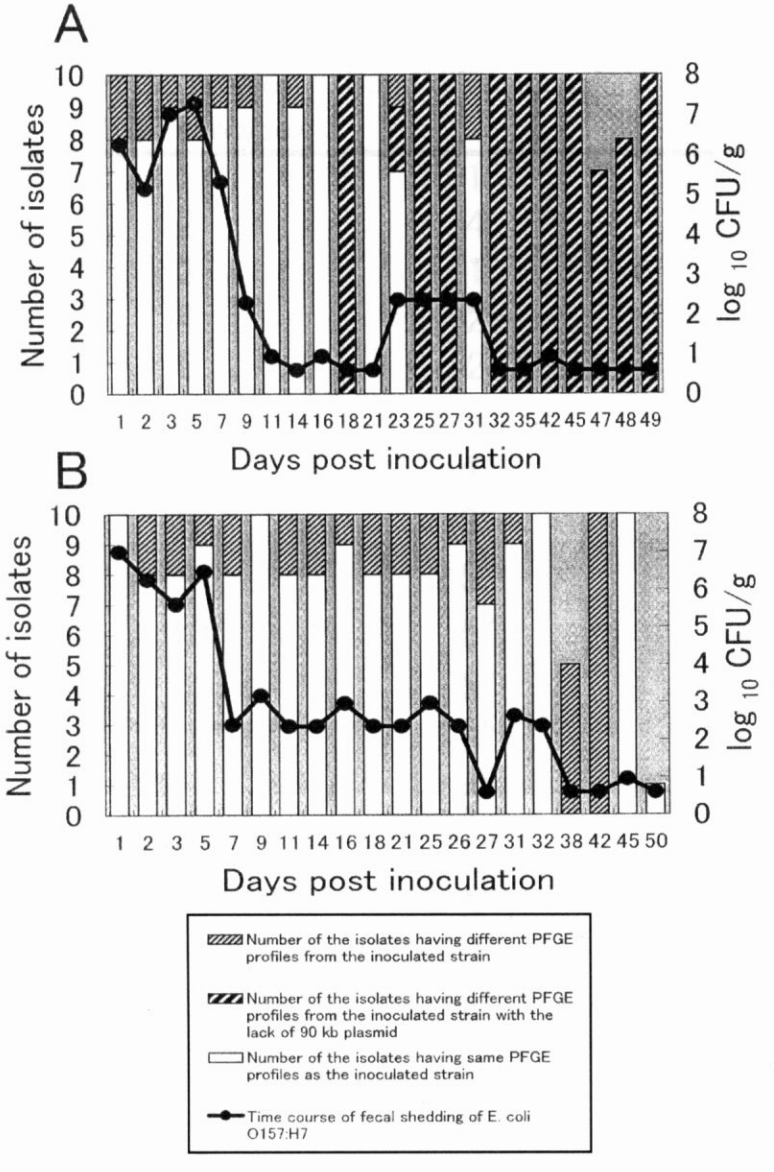

Fig. 6. Changes in PFGE profile and time course of fecal shedding of EHEC O157:H7 isolates from calf No. 1 (A) and 2 (B). The left vertical axis corresponds to the number of isolates (cumulative bar graph). The largest number was ten because up to ten isolates of EHEC O157:H7 were obtained from each selected media. The right vertical axis corresponds to the time course of fecal shedding ($\log_{10}$ CFU per gram of feces) of EHEC O157:H7 (sequential line graph). The data obtained on and after 9 days post inoculation from calf No. 1 and on and after 7 days post inoculation from calf No. 2 were determined by MPN. All the isolates recovered from calf No. 1 on and after 32 days post inoculation were mutated isolates. A manifest small peak of fecal shedding of EHEC O157:H7 was observed just prior to this change. All the isolates recovered from calf No. 2 on 38 and 42 days post inoculation were mutated isolates. A small peak of fecal shedding was also observed just prior to this change.

Table 10. List of the PFGE profiles different from that of the inoculated strain

Origin	PFGE profile (PFGE subtype)	Band differences[a]	No. of isolates
Calf No. 1	1	1	4
	2	4	1
	3	1	1
	4	2	1
	5	2	1
	6	1	2
	7	4[b]	10
	8	3[b]	1
	9	5[b]	10
	10	5[b]	1
	11	5[b]	5
	12	6[b]	1
	13	5[b]	66
	14	4[b]	1
	15	5[b]	1
	16	1	2
	17	6[b]	1
Calf No. 2	18	2	4
	19	1	5
	20	1	2
	21	1	1
	22	2	1
	23	2	17
	24	2	2
	25	1	4
	26	4	1
	27	2	1

[a] No. of bands of sizes different from those in the inoculated strain.

[b] Two of the band differences originated from the lack of 90 kb plasmid.

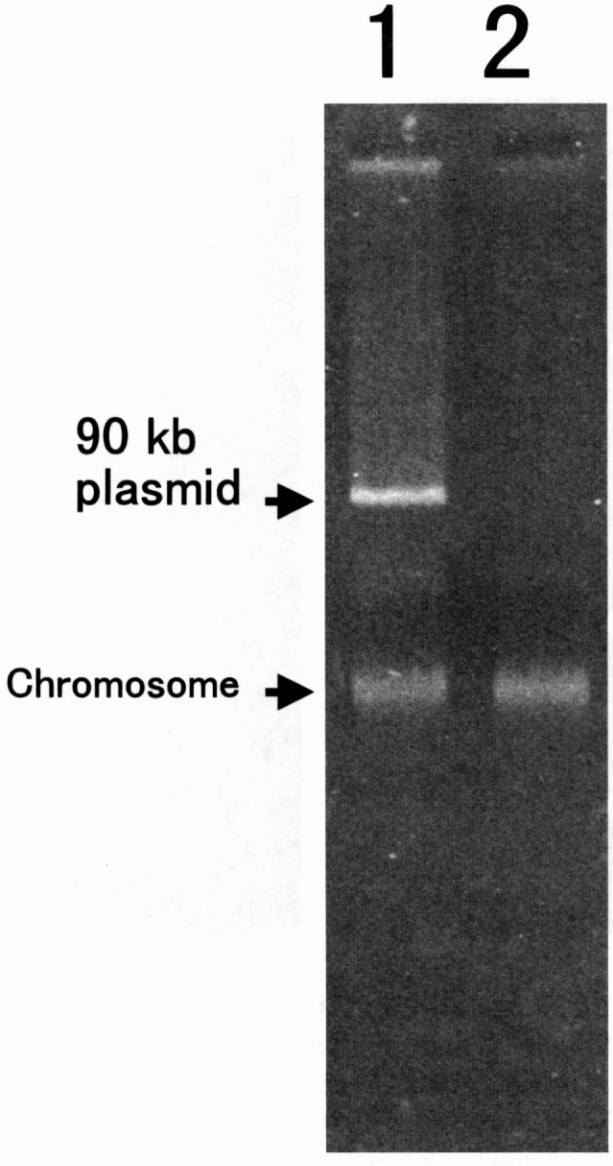

Fig. 7. Plasmid profiles of EHEC O157:H7 isolates. Lane 1 is the inoculated strain. Lane 2 is the plasmid lost EHEC O157:H7 isolate recovered from experimentally infected cattle.

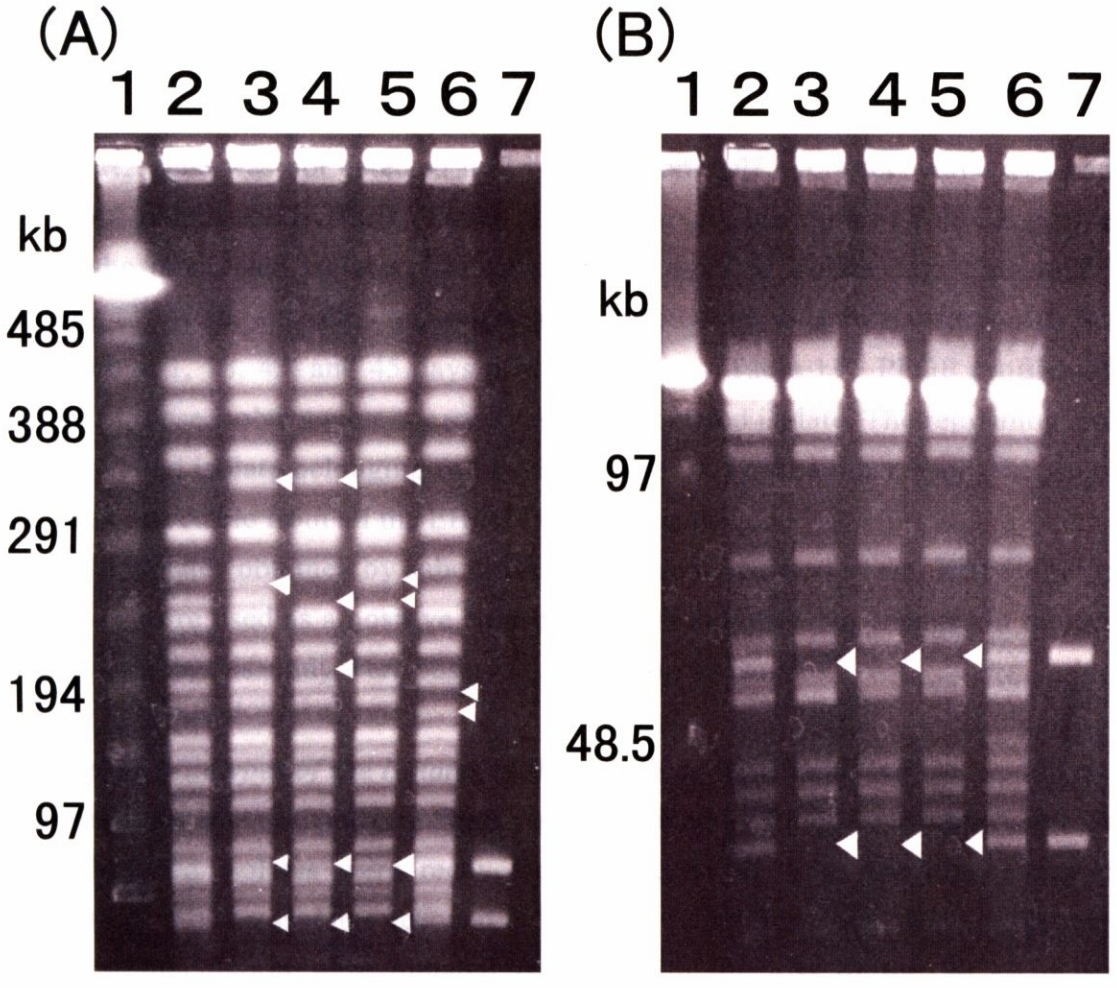

Fig. 8. PFGE patterns of EHEC O157:H7 isolates and its 90 kb plasmid. (A) For separation of whole genome, linearly ramped switching time from 15 to 40 s was applied for 24 h. Lane 1, Lambda ladder as a size marker; lane 2, EHEC O157:H7 strain inoculated; lane 3, PFGE subtype 7 isolate; lane 4, PFGE subtype 9 isolate; lane 5, PFGE subtype 13 isolate, lane 6, PFGE subtype 23 isolate; lane 7, 90 kb plasmid isolated from the injected EHEC O157:H7. White arrowheads indicate observed band differences. (B) For separation of fragments of less than 100 kb, a constant switching time of 4 s was applied for 20 h. The origin of each isolate is the same as that described for panel A. White arrowheads indicate band differences observed in less than 100 kb bands.

The loss of the 90 kb plasmid among mutated isolates recovered from calf No. 1 at 18 days post inoculation was observed (Fig. 7). None of the isolates recovered from calf No. 1 on and after 32 days post inoculation contained the 90 kb plasmid. Approximately 60 and 30 kb-sized bands on the PFGE profiles of these isolates were lost (Fig. 8). The result of PFGE of isolated 90 kb plasmid showed that these two bands are originated from the 90 kb plasmid (Fig. 8).

The *stx2* gene was not detected in 2 isolates from calf No. 1 recovered at 5 and 47 days post inoculation by PCR (Table 11). One isolate has the same PFGE profile as that of the inoculated isolates. The other was a mutated isolate belonging to PFGE subtype 13. Also, the *stx2* gene was not detected in 8 isolates from calf No. 2 recovered at 1, 18, 42, and 45 days post inoculation (Table 11). PFGE profiles of all but one of these isolates were the same as the inoculated isolate. The remaining isolate was a mutated isolate belonging to PFGE subtype 23. On the other hand, the gene for *eaeA* was detectable from all the EHEC O157:H7 isolates.

The 10 isolates obtained by subcultivation of the inoculated strain, EHEC O157:H7 strain 3-0, were used as a positive control for each analysis. No changes in molecular markers used in this study were observed among the 10 isolates.

Changes of genotypes of the isolates in the course of fecal shedding

In this study, the shift of genotypes, as determined by PFGE, was observed in both cattle. Mutated isolates were recovered from calf No. 1 as early as 1 day post inoculation. All the isolates recovered from the feces from calf No. 1 collected 18 days post inoculation were mutated isolates with the loss of the 90 kb plasmid. Though the isolates having the same PFGE pattern as the inoculated isolates were recovered on three sampling dates on and after 21 days post inoculation, all the isolates recovered from feces collected on and after 32 days post inoculation were mutated isolates with loss of the 90 kb plasmid (Fig. 6A). A small peak of fecal shedding of EHEC O157:H7 was observed from 23 to 31 days post inoculation (Fig. 6A). Mutated isolates were also recovered from calf No. 2 as early as 2 days post inoculation. From 1 to 3 mutated isolates were isolated from feces collected 13 of 16 sampling dates up to 32 days post inoculation. All the isolates recovered at 38 and 42 days post inoculation were mutated isolates belonging PFGE subtype 23 (Fig. 8), while the PFGE patterns of all the isolates recovered at 45 and 50 days post inoculation were the same as that of the inoculated isolates (Fig. 6B). A small peak of

Table 11. List of the *stx2* gene negative isolates by PCR

Origin	Serial No.	Date of isolation (Days post inoculation)	PFGE profile
Calf No. 1	1	5	Inoculated[a]
	2	47	Mutated (13)[b]
Calf No. 2	1	1	Inoculated
	2	18	Inoculated
	3	42	Mutated (23)
	4	45	Inoculated
	5	45	Inoculated
	6	45	Inoculated
	7	45	Inoculated
	8	45	Inoculated

[a]A strain having same PFGE profile as the inoculated strain.

[b]A strain having different PFGE profile from the inoculated strain. The number in the parenthesis indicates the PFGE profile number listed in Table 10.

fecal shedding of EHEC O157:H7 was also observed on 32 days post inoculation (Fig. 6B).

Discussion

Genetic diversity among EHEC O157:H7 isolates has been reported (16, 26, 29, 43). This diversity is produced by mutation, for one explanation (27). Specifically, several groups have reported instability of molecular markers of EHEC O157:H7. The genes for *stx1* and *stx2* were carried on bacteriophages, and the loss of the *stx* gene with changes of PFGE profile have been reported (19, 33). The same phenomenon was also observed in EHEC O157: H7 isolates from naturally infected cattle in Chapter II. However, specific changes in PFGE profile were not observed in association with the loss of the *stx2* gene in this study. On the other hand, the gene for *eaeA* was detectable from all the EHEC O157:H7 isolates suggesting the gene for *eaeA* is relatively stable.

Of the 27 novel PFGE profiles, the number of bands of sizes different from those in the inoculated strain was six or fewer. Two of the six band differences were originated from the loss of the 90 kb plasmid. The results that the up to four band differences from the chromosome origin was observed should be considered when undertaking an epidemiological survey using PFGE.

The shift of genetic subtypes of EHEC O157:H7 was also observed in two experimentally infected cattle as well as naturally infected cattle on farm in Chapter II. All the EHEC O157:H7 isolates obtained on and after 9 days post inoculation were isolated from enrichment culture, so the possibility that the author detected the growth of a unique clone can not be denied. But the aouthor never detected any isolates having the same PFGE profile as that of inoculated strain on and after 32 days post inoculation from calf No. 1. The possibility of concurrent infection with multiple genotypes can be ruled out in this experimental environment, and no changes in the molecular marker of the 10 isolates obtained by subcultivation of the inoculated strain were observed. So it can be considered that this phenomenon reflected a genetic change and clonal replacement within the calves. On the other hand, it should be noted that there was a small peak of fecal shedding of EHEC O157:H7 in each calf just prior to the shift of genotypes (Fig. 6). These peaks of fecal shedding of EHEC O157:H7 may indicate the proliferation of mutated isolate within the calves.

Karch *et al.* (19) used PFGE to compare the first and last EHEC O157:H7 isolates from long-term shedders. In three of the seven human patients, PFGE types of the isolates were varied. They referred to this phenomenon as clonal turnover, which refers to "dramatic changes in clonal composition characterized by the appearance of

new clonal genotypes and loss of old clones." The shift of genotypes of EHEC O157:H7 isolates in calf No. 1 was in agreement with this definition. This is the first report describing the clonal turnover of EHEC O157:H7 within cattle. No specific characters except for molecular markers of new clones from calf No. 1 have been observed to date. The exact nature of the mechanism governing the clonal turnover of EHEC O157:H7 within cattle remains unclear.

The duration of EHEC O157:H7 colonization within cattle is typically 2 months or less (13). On the other hand, Shere *et al.* (46) reported that EHEC O157:H7 strains can persist in a herd for a 2-year period based on the results of a longitudinal study on four dairy farms. They pointed out that the fecal shedding of this agent in cattle was intermittent and may result from re-inoculation from environmental sources rather than colonization. Though the fecal shedding of EHEC O157:H7 was also observed for a relatively short period, about 50 days in this study, the shift of genotypes of EHEC O157:H7 was observed within both calves. These results indicate that multiple EHEC O157:H7 genotypes with relatively high similarity can exist in one farm at the same time, and the diversity of the genotype of this agent is amplified in the course of its persistence on a farm. Some reports have corroborated this possibility (9, 21, 43, 46, 51). Replication and dissemination of the EHEC O157:H7 strains mutated in cattle by clonal turnover may lead to the diversification of this agent among cattle populations.

Summary

The duration of EHEC O157:H7 colonization within two experimentally infected calves were about 50 days. EHEC O157:H7 was detectable from both calves only by enrichment culture on and after 9 days post inoculation. A total of 401 EHEC O157:H7 isolates from two experimentally infected calves were analyzed using molecular biological methods. Genetic differences detected by PFGE were observed between the inoculated and recovered strains as early as 1 day post inoculation. Up to 4 band differences from the chromosome origin among the mutated isolates were observed. The loss of the inoculated clone was observed in one calf. Replication and dissemination of the EHEC O157:H7 strains that mutated in cattle may result in the diversification of this organism among cattle populations.

Chapter Ⅳ

A comparison of EHEC O157 isolates from cattle in Japan and the USA by molecular biological methods

Introduction

Chapter Ⅰ showed the results of molecular typing of EHEC O157:H7 isolates from cattle in Japan. Fifteen phage types and 50 PFGE profiles were observed in the bovine isolates. These results indicate that EHEC O157:H7 strains with various genetic subtypes have already spread to cattle in Japan. One explanation for this diversity is that exchange of this agent between Japan and other countries through common reservoirs has added to the background of genetic diversity produced by replication and dissemination of the EHEC O157:H7 strains mutated in cattle by clonal turnover shown in ChapterⅢ. If so, there would be identical or closely related EHEC O157:H7 from cattle raised in places geographically wide apart, such as Japan and the USA. In order to test this hypothesis, we compared EHEC O157:H7 isolates from cattle in Japan with those from cattle in the USA by using molecular methods.

Materials and Methods

Strains

A total of 91 EHEC O157:H7 (H-) isolates from 91 cattle in Japan (Japanese isolates) were primarily obtained from a previous investigation explained in Chapter I . Seventy-seven of these isolates were isolated from fecal samples from August to October in 1996 at regional livestock hygiene service centers of 23 prefectures. Fourteen isolates were obtained from fecal samples from 3 prefectures in 1996 and 1997. A total of 415 *E. coli* O157 isolates from 415 fecal samples from cattle in the USA (US isolates) were also obtained from a previous investigation. Three hundred and three isolates were isolated from 41 different farms in the states of Washington, Oregon and Idaho during 1991 to 1995 (43). A hundred and twelve isolates were obtained from 31 feedlots located throughout the USA in 1994 (12). None of these US isolates were assayed for the H flagellar antigen.

Comparison of Japanese and US isolates by cluster analysis

PFGE profiles using *Xba* I (Gibco-BRL, Gaithersburg, MD, USA) digested genomic DNA were initially obtained as described in Chapter I . Each PFGE profile was compared with all of the others by a previously described method (43). Briefly, a restriction fragment length polymorphism (RFLP) analysis software program (DNA ProScan PRO-RFLP, Nashville, TN, USA) was used to estimate the sizes of the largest 9 bands of DNA by using scanned images of photographs. A SAS clustering procedure (SAS Institute, Cary, NC, USA) was then used to designate the possible indistinguishable profiles, and comparison of individual isolates within clusters was performed by a spreadsheet program that utilized the data on DNA band size.

Comparison of the isolates on the same PFGE gels

Xba I -cleaved DNA from Japanese isolates that had identical profiles to US isolates by this procedure was compared by running them together on agarose gels using three kinds of switching times to fractionate and compare restriction fragments (Fig. 9): (i) $<$600 kb, ramped from 5 to 50 s for 24 h; (ii) $<$300 kb, ramped from 10 to 20 s for 22 h; and (iii) $<$100kb, constant switching times of 4 s for 20 h. The isolates having similar profiles in all sized bands were further compared by PFGE using additional restriction endonucleases *Bln*

I (Boerhinger Mannheim, Mannheim, Germany) and *Spe* I (Promega, Madisaon, WI, USA). Restriction endonuclease digestion was performed according to the manufacturer's protocol. The pulse time was increased from 5 to 50 s over 24 h for *Bln* I -digested DNAs and 10 to 20 s over 22 h for *Spe* I -digested DNAs.

Comparison of the isolates by toxin gene location and phage type

The aouthor also compared the toxin gene location on the PFGE profiles of *Xba* I -cleaved DNA of these isolates by Southern blot analysis probed by the *stx1* and *stx2* genes. *Xba* I -cleaved DNA of these isolates were transferred to positively charged nylon membranes (Boerhinger Mannheim) in 0.4 M NaOH solution. A 471 bp DNA fragment of the *stx1* gene and of the *stx2* gene were amplified with the primer pairs described by Kobayashi (23), and with DNA from EHEC O157:H7 NIID 212 strain, which possesses both *stx1* and *stx2* genes. This strain, isolated from patient in the large outbreak in Sakai city, Osaka prefecture, Japan, in 1996, was kindly provided by the National Institute of Infectious Diseases of Japan (Table 4). The amplified fragments were labeled with digoxigenin (DIG)-11-dUTP. Probe labeling, hybridization and detection of filter-bound labeled DNA were performed according to the manufacturer's protocol (DIG DNA Labeling and Detection Kit, Boerhinger Mannheim).

Phage typing of these isolates was performed by the procedure described in Chapter I. Dr. Takashi Masuda of SPIPHE carried out this experiment.

Results

Twenty-eight of 91 Japanese isolates had identical profiles to 69 US isolates using the largest 9 bands. Detailed comparison of these isolates was performed by the PFGE on the same gels, toxin gene location, and phage type. Figs. 9 and 10 show the examples of comparison by PFGE and toxin gene location, respectively. Three of 28 Japanese isolates were very similar to at least one of the US isolates (Table 12). The numbers of band differences between Japanese and US isolates in the PFGE profiles of *Xba* I -cleaved genomic DNAs were less than 6 (Table 12). By *Bln* I digestion, the isolate JP37 was indistinguishable from the isolate US1914 (Table 12). JP102 was indistinguishable from the isolates US1412, US1665 and US1814 by *Spe* I digestion (Table 12). The toxin gene location of the isolates JP37 and JP88 were the same as that of the isolates US1914 and US1920, respectively (Table 12). Moreover, the phage types of the isolates JP88 and JP102 were the same as the isolates US1920 and US1412, respectively (Table 12).

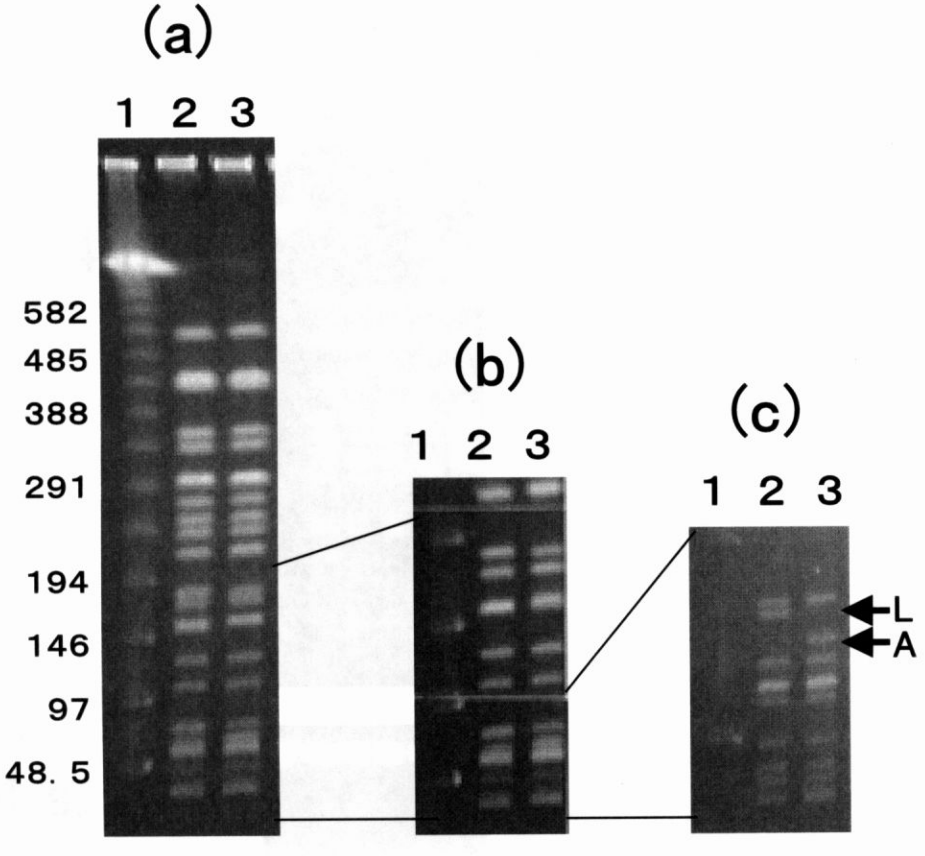

Fig. 9. Comparison of *E. coli* O157 isolates from cattle in Japan and the USA by PFGE using *Xba* I -cleaved genomic DNAs. (a) Switching times ramped from 5 to 50 s for 24 h was used to compare all sized bands. (b) Switching times ramped from 10 to 20 s for 22 h was used to compare $<$300 kb sized bands. (c) Constant switching times of 4 s for 20 h was used to compare $<$ 100 kb sized bands. In (a), (b), and (c), lane 1 is a Lambda ladder as a size marker. Lane 2 is a profile of the Japanese isolate JP37. Lane 3 is a profile of the US isolate US1914. L indicates a lack of the band and A indicates an additional band in the profile of the US isolate compared to that of the Japanese isolate. In this case, 2 band differences were determined.

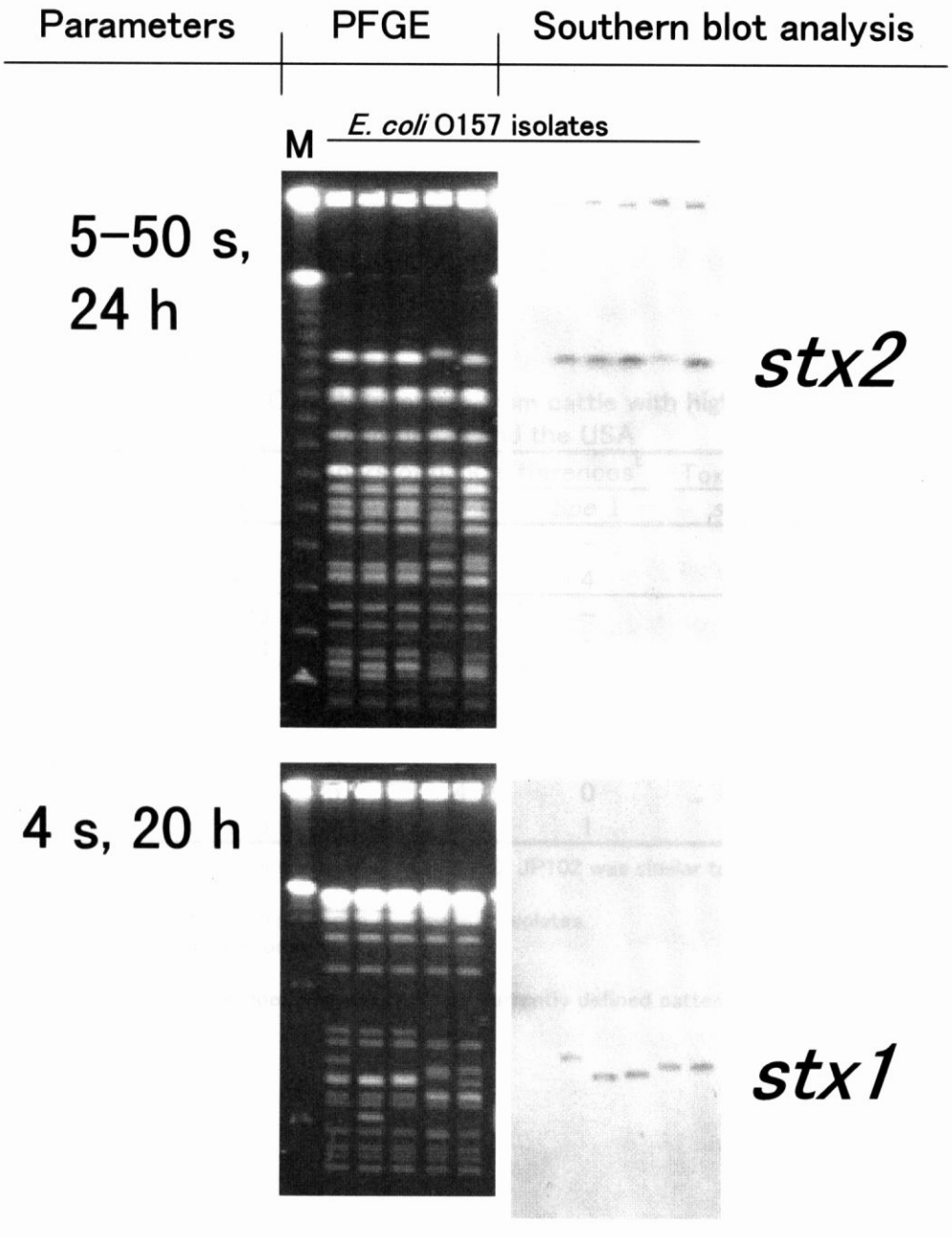

Fig. 10. Determination of the toxin gene location on PFGE profiles of *E. coli* O157 isolates by Southern blot analysis. Left and central columns indicate the parameters and results of PFGE, respectively. Right column indicates the hibridization results by each stx gene specific probes. M indicates a Lambda ladder as a size marker. Other lanes are the profiles of EHEC O157:H7 isolates from cattle in Japan and the USA.

Table 12. EHEC O157:H7 isolates from cattle with high similarity between Japan and the USA

Isolates[a]	Origin	Date of isolation	Numbers of band differences[b]			Toxin gene location[c]		PT[d]
			Xba I	*Bln* I	*Spe* I	*stx1*	*stx2*	
JP37	Japan	Aug, 1996	–	–	–	70	530	21
US1914	USA	Nov, 1994	2	0	4	70	530	32
JP88	Japan	Sep, 1996	–	–	–	–	130	54
US1920	USA	Nov, 1994	2	3	1	–	130	54
JP102	Japan	Jun, 1997	–	–	–	70	520	26
US1412	ID, USA	Sep, 1994	5	3	0	70	530	26
US1665	WA, USA	Oct, 1994	5	2	0	70	530	32
US1814	WA, USA	Nov, 1994	5	4	0	48	530	RDNC[e]
US3220	ID, USA	Sep, 1995	4	2	1	70	530	14

[a] JP 37 was similar to US1914. JP88 was similar to US1920, JP102 was similar to US1412, US1665, US1814, and US3220.
[b] Number of band of sizes different from those in Japanese isolates.
[c] Size of band where stx genes are located.
[d] Bacteriophage types.
[e] Isolate reacts typing phages but does not conform to a currently defined pattern.

Discussion

Chapter I showed that PFGE has a sufficient discriminatory ability to subtype bovine EHEC O157:H7 isolates in Japan and can be used as a powerful epidemiologic tool. The number of band of sizes different from that in each other was more than 15 in samples with similarity coefficient of less than 0.40 (Fig. 1). According to guidelines proposed by Tenover *et al.* (47), 6 or fewer band differences indicate a possible genetic relationship between two isolates. However, the authors restricted the use of these criteria to analyzing small sets of isolates collected over relatively short periods of time in hospitals or communities. They recommended the use of multiple restriction endonucleases and other analyses in investigations for potential genetic relationships among isolates collected over extended periods. On the other hand, up to four band differences from the chromosome origin between the inoculated and recovered *E. coli* O157 isolates from experimentally infected cattle were observed (see Chapter Ⅲ). Four or fewer band differences on PFGE profiles of *E. coli* O157 isolates from cattle may indicate the possibility that the isolates share the same origin.

Because the Japanese and the US *E. coli* O157 isolates had been collected over a period of 7 years and from a wide area, the author compared them directly by PFGE using three kinds of endonucleases. The author found that 3 of 28 Japanese isolates were very similar to US isolates. The numbers of band differences were 5 or less using any enzyme. Moreover, the results from toxin gene location and phage typing suggested a close relation especially between the isolates JP88 and US1920. Thus these Japanese and US isolates may share the same origin. Common reservoirs of colonization for cattle farms may have existed in Japan and the USA.

Rice *et al.* (43) reported that identical subtypes of *E. coli* O157 isolates were found on farms separated by as much as 640 kilometers. They suggested the possibility that *E. coli* O157 can be transmitted inter-regionally to cattle farms. They also suggested that cattle feed can be a vehicle for this transmission. Although *E. coli* O157 has not been reported to have been found in marketed cattle feeds (30), our finding of *E. coli* O157 isolates with high similarity from cattle in Japan and the USA supports this possibility. EHEC O157:H7 may invade cattle populations presumably through feedstuffs and/or farm animals.

Summary

E. coli O157 isolates from cattle in Japan (n=91) and in the USA (n= 415) were compared by PFGE of endonuclease-cleaved genomic DNA, location of the *stx* genes and phage typing. Three isolates from cattle in Japan with high similarity to isolates from cattle in the USA were found. Isolates from cattle farms in Japan and the USA may share a common source.

Conclusion

The goal of this study was to gain insight into the ecology and mode of transmission of EHEC O157:H7 among the cattle populations from the aspect of a possible source of human EHEC O157:H7 infections.

In order to conduct an epidemiological study of EHEC O157:H7 among cattle populations, suitable epidemiological marker for discrimination of the isolates needed to be found. First of all, an evaluation of epidemiological markers was performed. A total of 77 isolates were investigated by molecular biological methods. Most of these isolates (43 isolates) possessed the *stx2* gene but not *stx1*. Fifteen phage types and fifty PFGE profiles were observed. These results indicate that EHEC O157:H7 strains with various genetic subtypes have already spread to cattle in Japan.

Of the three methods of subtyping EHEC O157:H7, PFGE was considered the best from the aspects of discriminatory power and reproducibility. PFGE can be used as a powerful epidemiologic tool. The discriminatory power of phage types was relatively high, but the results of phage typing and PFGE subtyping were discordant. These results suggest that a combined use of phage typing and PFGE would provide a more detailed classification of the isolates.

One EHEC O157:H7 isolate from cattle in Japan was indistinguishable from the human outbreak strain in terms of toxin profile, phage type, and PFGE profile. This indicates that cattle must be considered a possible source of human EHEC O157:H7 outbreaks in Japan as well as in North America and Europe.

Next, the ecology of EHEC O157:H7 among cattle populations on farms was investigated. The duration of EHEC O157:H7 colonization within cattle was about 50 days in experimentally infected cattle. Clonal turnover, that is, genetic change and clonal replacement, was often observed within cattle in the course of colonization. Up to four band differences from the chromosome origin between the inoculated and recovered EHEC O157:H7 isolates from experimentally infected cattle were found. This observation should be considered when undertaking an epidemiological survey using PFGE. Similarity, rather than exact matching, arguably gives a better understanding of the outbreak of EHEC O157:H7. This is the first report describing the clonal turnover of EHEC O157:H7 within cattle.

Finally, EHEC O157:H7 isolates from cattle in Japan and the USA were compared by using molecular biological methods to discuss the epidemiology of this agent from a global viewpoint. Three isolates from cattle in Japan with a high similarity to isolates from cattle in the USA were found. Isolates from cattle farms in Japan and the USA may share a common source.

In summary, it was suggested that PFGE has a sufficient discriminatory power to subtype bovine EHEC O157:H7 isolates in Japan and can be used as a powerful epidemiologic tool. One EHEC O157:H7 isolate from cattle in Japan was indistinguishable from the human outbreak strain in terms of toxin profile, phage type, and PFGE profile. In addition, the duration of EHEC O157:H7 colonization within cattle was relatively long period of about 50 days or more. These data indicate that cattle must be considered a possible source of human EHEC O157:H7 outbreaks in Japan as well as in North America and Europe. Clonal turnover, that is, genetic change and clonal replacement detectable by PFGE, was often observed within cattle in the course of colonization. Moreover, three isolates from cattle in Japan with high similarity to isolates from cattle in the USA were found. Isolates from cattle farms in Japan and the USA may share a common source.

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Summary in Japanese

論文の内容の要旨

論文題目　Molecular epidemiological study on enterohemorrhagic *Escherichia coli* O157:H7 isolates from cattle using pulsed-field gel electrophoresis
(パルスフィールドゲル電気泳動を用いた牛由来腸管出血性大腸菌 O157:H7 に関する分子疫学的研究)

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はじめに

腸管出血性大腸菌 O157:H7 (O157) は，1982 年に米国において集団食中毒の原因と認識されて以来世界的な問題となり，わが国においても 1996 年に本菌による集団食中毒が多発した。北米やヨーロッパにおいては O157 保菌牛が人の感染源として重要視されているが，わが国においては人の集団感染に牛が直接関連した事例は報告されていない。一方，農林水産省が 1996 年に行った農場における O157 の浸潤調査では 0.62%の牛から本菌が分離されている。しかし，これら保菌牛の排菌数や排菌期間に関する情報は少なく，農場レベルでの本菌の動態は不明であることから，人の O157 感染症におけるわが国の牛群の重要性は依然として明らかでない。そこで本研究においては，農場における O157 の生態と伝播様式の解明を目的として一連の研究を行った。

1．わが国の牛から分離された O157 の遺伝子型別

農場レベルでの O157 の疫学的動態を明らかにするために必要な O157 型別法の評価を行った。23 県の家畜保健衛生所等で 77 頭の牛から分離した 77 株の O157 について，パルスフィールドゲル電気泳動 (PFGE) 法による型別，PCR 法による毒素遺伝子型別，ファージ型別を行った。また得られた PFGE 像を用いてクラスター解析を行い，PFGE 型と毒素遺伝

子型，ファージ型との相関を調べた。これらの手法を用いて国立感染症研究所から分与を受けた人由来11株と牛由来株を比較した。

制限酵素 *Xba* I を用いたPFGEでは77株に50の異なる泳動像を認めた。ファージ型別では15の型が確認され，このうちファージ型21（16株，20.8％），54（16株，20.8％），および34（11株，14.3％）が優勢であった。また77株中75株（97.4％）は *stx1* および *stx2* のいずれかの遺伝子を保有しており，28株（36.4％）は両遺伝子保有菌，43株（55.8％）は *stx2* 単独保有菌であった。

識別力の指標であるシンプソンのD値はPFGE型別で0.987，ファージ型別で0.884，すなわち77株からランダムに2株を選んだとき，それらが異なる型に属する確率がPFGEで98.7%，ファージ型別で88.4%と算出された。したがって，わが国の牛由来O157型別法としてはPFGEが識別力の点で優れており，疫学解析上の有力なツールとして使用可能であると考えられた。一方，PFGE像を用いてクラスター解析を実施したところ，PFGE型と毒素遺伝子型には相関が認められたが，PFGE型とファージ型とは関連性が低かった。したがって牛由来O157型別法としてPFGEを標準的な方法として用い，ファージ型別を併用することによって，より詳細な疫学的知見を得ることができるものと考えられた。

また牛由来77株中1株は2種類の制限酵素（*Xba* I，*Bln* I）を用いたPFGE，毒素遺伝子型別，ファージ型別のいずれの手法を用いても人の集団感染株と識別不能であった。これは，欧米と同様にわが国においても牛が人への感染源となっている可能性を示唆する成績と考えられた。

2．自然感染牛におけるO157の排菌パターンと分離菌の遺伝子型の変化

農場における本菌の排菌数，排菌期間を究明するために，1農場における保菌牛7頭について経時的に糞便からの菌分離を行い，分離菌の毒素遺伝子型やPFGE像を調べた。7頭中1頭において2ヶ月以上の持続的排菌が，3頭において2ヶ月前後の間欠的排菌がそれぞれ認められ，この間の排菌数は糞便1g当たり43個以下と推定された。分離菌46株をPFGEで解析したところ，*Xba* Iで4型，*Bln* Iで6型，*Spe* Iで4型に区分することができ，さらにこの組み合わせで，46株を9つのサブタイプに区分することができた。1菌株を除くと，各制限酵素で検出できた異なるバンドの数は3本以下であり，これら菌株の近縁度が高いことが示された。

調査対象牛のうち持続的または間欠的排菌が認められた4頭中3頭において分離菌のサブタイプが時間の経過とともに変化する現象が認められた。特に保菌牛1頭においては，1週間おきに分離菌のサブタイプが変化した。これら分離菌は互いに近縁であることが示唆されたので，本現象は牛腸管内における遺伝的変異と優勢菌の交代を反映するものと推察された。

3．実験感染牛における O157 の排菌パターンと分離菌の遺伝子型の変化

自然感染牛で認められた O157 分離菌の遺伝子型変化が実験的に再現できるか否か検討を加えた。事前に腸管出血性大腸菌陰性であることを確認した８週齢のホルスタイン去勢雄牛３頭を個別飼育し，うち２頭（No. 1，2）に 10^9CFU の O157（*stx1*－，*stx2*＋，*eaeA*＋）を経口投与し，他の１頭（No. 3）は未投与対照とした。投与の翌日より計３頭の牛から毎日直腸便を採取し，O157 を分離し，それを PFGE で解析した。

図１に示したように２頭の実験感染牛において，O157 の排菌は投与後 49 日，50 日後まで観察された。未投与対照牛からは O157 は分離されなかった。自然感染牛における成績と合わせて考えると，O157 は牛に対して病原菌としてよりも，大部分の非病原性大腸菌と同様の挙動を示し，その排菌は再感染がなければ２ヶ月前後で終了することが示唆された。

分離菌 401 株の解析において，投与後２日目には両牛から変異菌が回収された（図１）。牛 No. 1 では投与後 32 日目以降投与菌が回収不能となった。牛 No. 2 では投与後 38，42 日目に投与菌は回収されなかったが，その後投与菌のみが回収された。各牛由来の変異菌に 17 種類および 10 種類の PFGE 型が認められたが，両牛間で同じ PFGE 型を示す株は認められなかった。以上の成績から牛腸管内における O157 のランダムな遺伝子型の変化と適応遺伝子型の増殖による優勢菌の交代を反映する現象が２頭の実験感染牛で再現できたものと考えられた。

牛 No. 1 から分離された株に 90 kb プラスミドの脱落を伴う変異菌が認められた（図１）。プラスミド脱落による変化を差し引くと，変異菌と投与菌の PFGE 像の比較において，染色体 DNA に由来する範囲で異なるバンド数は最大４本であった。このことから，PFGE を用いて牛由来 O157 の解析を行う場合，少なくとも異なるバンド数が４本までは疫学的関連を疑う必要があることが示唆された。

4．日本および米国の牛から分離された O157 の疫学マーカーによる比較

グローバルな視点からの疫学的知見を得ることを目的として，わが国と米国における牛由来 O157 の比較を行った。国内の牛から分離された 91 株と米国内で牛から分離された 415 株を PFGE，PCR，あるいはファージタイピング等の手法によって比較したところ，日本の牛から分離された３株（JP37，JP88，JP102）は米国の牛から分離された株と高い相同性を示した。すなわち JP37 株は１米国株と *Bln* I を用いた PFGE で識別不能であり，*Xba* I，*Spe* I を用いた PFGE では異なるバンド数が４本以下であった。また JP88 株は１米国株と同じファージ型に属しており，上記３種の制限酵素を用いた PFGE で異なるバンド数は３本以下であった。さらに JP102 株は１米国株と同じファージ型に属しており，上記３種の制限酵素を用いた PFGE で異なるバンド数は５本以下であった。以上の成績から本菌は共通の媒体を介して長い距離を伝播し，複数の大陸の牛群に広がる可能性が考えられた。

おわりに

以上の研究より，わが国の牛由来O157型別法としてはPFGEが識別力の点で優れており，疫学解析上の有力なツールとして使用可能であることが明らかとなった。牛由来77株中1株は2種類の制限酵素を用いたPFGE，毒素遺伝子型別，ファージ型別のいずれの手法を用いてもヒトの集団感染株と識別不能であったこと，さらに牛の保菌期間が少なくとも50日以上であったことから，わが国においても牛が人への感染源となっている可能性が示唆された。また本研究において牛の腸管内でO157の遺伝子型の変化と優勢遺伝子型の交代現象が起こることを初めて明らかにした。さらにO157は共通の媒体を介して長い距離を伝播し，複数の大陸の牛群に広がる可能性が考えられた。

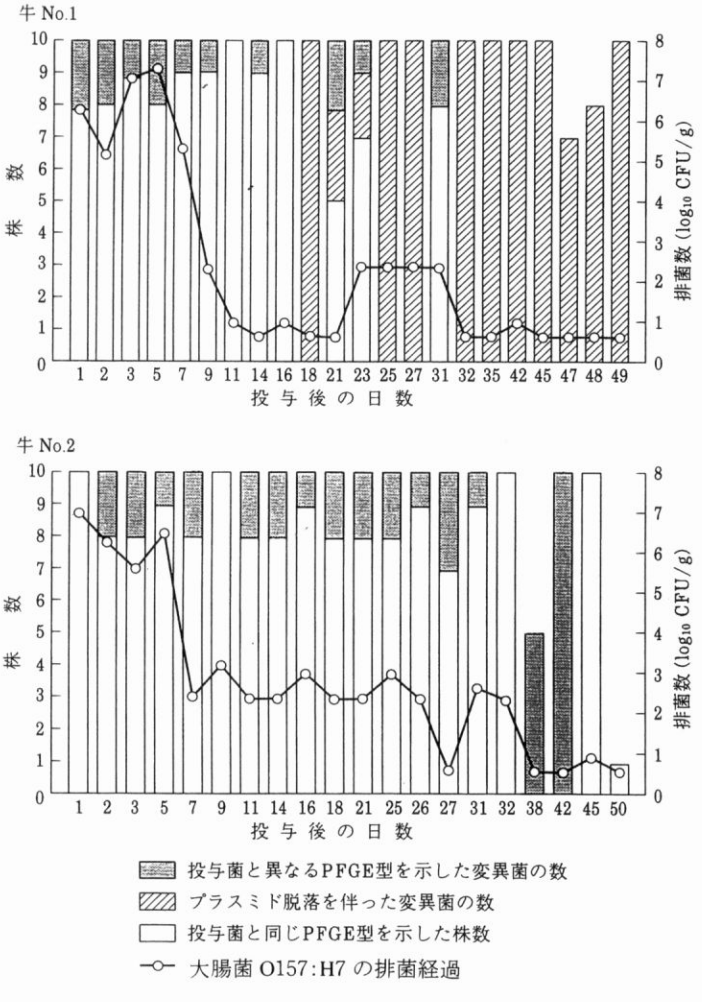

図1　実験感染牛における大腸菌O157：H7の排菌経過と分離菌のPFGE像の変化