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Supplementary data

Supplementary data (^{1}H NMR data) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.03.057.

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FULL PAPER *Bacteriology*

Evaluation of a PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) Assay for Molecular Epidemiological Study of Shiga Toxin-Producing *Escherichia coli*

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ABSTRACT. In this study, we have evaluated our recently developed polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay for the molecular subtyping of Shiga toxin-producing *Escherichia coli* (STEC). A total of 200 STEC strains including O157 (n=100), O26 (n=50), O111 (n=10), and non-O26/O111/O157 (n=40) serogroups isolated during 2005–2006 in Japan, which were identified to be clonally different by pulsed-field gel electrophoresis (PFGE) were further analyzed by the PCR-RFLP assay in comparison to PFGE. Ninety-five of O157, 48 of O26, five of O111 and 19 of non-O26/O111/O157 STEC strains yielded one to three amplicons ranging from 6.0 to 15.5 kb in size by the specific primer set targeting region V which is located in the upstream of *stx* genes. These strains were classified into 41 (O157), 8 (O26), 4 (O111) and 17 (non-O26/O111/O157) groups based on the RFLP patterns obtained by subsequent restriction digestion, respectively. Although the discriminatory power of PCR-RFLP assay was somewhat less than that of PFGE, it is more convenient for molecular subtyping of STEC strains especially for O157, the most important serogroup implicated in human diseases, as well as to identify the outbreak-associated isolates because of its simplicity, rapidity, ease and good reproducibility.

KEY WORDS: *E. coli*, molecular epidemiology, molecular typing, PCR-RFLP, STEC.

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Shiga toxin-producing *Escherichia coli* (STEC) has emerged as an important food-borne pathogen infecting thousands of people every year in Japan, and US [22]. Shiga toxin (Stx), the prime virulence factor of STEC, is classified into two groups namely Stx1 and Stx2 on the basis of their immunological properties [38]. Both Stx1 and Stx2 are encoded on lambda-like bacteriophage, so-called Stx-phage [38, 39]. Although more than 400 serotypes of STEC strains have been reported, O157:H7 is the most important serotype associated with human diseases such as hemorrhagic colitis and hemolytic-uremic syndrome [32, 38, 39] and is also the most predominant serotype isolated from sporadic cases and outbreaks [11, 45]. Since most of the food-poisonings caused by STEC are related to the consumption of beef or beef products, cattle have been considered as a major source of infection [5]. Other vehicles such as contaminated water, vegetables, and fruits have also been considered to be infection source of STEC [1, 4, 33]. Furthermore, person-to-person transmission is also an important factor for STEC infection [13, 31].

Several molecular subtyping methods such as ribotyping, randomly amplified polymorphic DNA-PCR, pulsed-field gel electrophoresis (PFGE), multi-locus sequence typing (MLST), multiple-locus variable-number of tandem repeats analysis (MLVA) etc. have been developed and utilized for molecular epidemiological studies of STEC [6, 10, 21, 28, 29, 44]. Among these, PFGE is the most commonly used molecular typing method to identify the possible source and route of infection, and has been used for a variety of pathogens including STEC because of its high discriminatory power to reveal their clonal relationships [3, 40]. However, PFGE has some disadvantages [13, 15, 23, 35]. For instance, PFGE requires expensive and elaborate equipment, and skilled labor. PFGE is labor-intensive and fairly time-consuming. PFGE profiles of some strains cannot be analyzed because of smeared profiles, which may be associated with degradation of genomic DNA due to free radical produced during electrophoresis [8, 18, 37]. PFGE pattern of a single isolate of STEC could be altered by repeated subcultures *in vitro* [13, 36]. Passage through bovine or human gastrointestinal tract could also cause variation in PFGE patterns for STEC strains [2, 15]. Thus, there is a chance of misinterpretation of clonality of the same strain. In addition, it is not possible to handle large number of samples at a time [16].

Sato *et al.* identified 6 characteristic regions (I to VI) in Shiga toxin phage genome [33]. Among them, region V, which is located in the upstream region of the Stx2 operons, was identified to be the most distinct portion in the entire phage genome and may be a good target for molecular sub-

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typing of STEC strains. For this reason, we have developed a rapid and simple DNA fingerprinting method, PCR-restriction fragment length polymorphism (PCR-RFLP) assay based on the nucleotide sequence diversity within the region V of Stx-phage, for molecular epidemiological study of STEC strains [34–37]. In the present study, we have evaluated the PCR-RFLP assay targeting the region V of Stx-phages by using a variety of defined STEC strains including O157 and non-O157, which were identified to be clonally different by PFGE.

MATERIALS AND METHODS

Bacterial strains and growth media: A total of 200 STEC strains of various serogroups, including O157 (n=100), O26 (n=50), O111 (n=10) and non-O26/O111/O157 (n=40) were randomly selected from 1,795 strains which were provided from the prefectural and municipal health centers and public health institutes (PHIs) in Japan during 2005 to 2006. Serology was determined and all strains were identified to be different clone by PFGE at the National Institute of Infectious Diseases under the National Epidemiological Surveillance of Infectious Diseases undertaken in compliance with the Law Concerning the Prevention of Infectious Diseases and Medical Care for Patients of Infections [25, 26]. STEC strains used in this study were cultured either on L-agar or in L-broth.

Chemicals and enzymes: Chemicals were purchased either from Nacalai Tesque (Kyoto, Japan), Wako Pure Chemical Industries (Tokyo, Japan), or Sigma Chemical Co. (St. Louis, MO, U.S.A.). Restriction enzymes, Takara LA Taq, and LA PCR kit version 2 were purchased from Takara Bio (Shiga, Japan). Bacto tryptone and yeast extract were purchased from Becton, Dickinson and Company (Franklin Lakes, NJ, U.S.A.). Pulsed-field certified, low-melting point preparative-grade, Seakem GTG and Seakem HGT (for high gelling temperature) agaroses, were either from Bio-Rad Laboratories, Inc. (Hercules, CA, U.S.A.) or Takara Bio. Molecular weight makers were purchased from Takara Bio.

Detection of stx1 and stx2 genes by multiplex PCR: Presence of *stx1* and *stx2* genes was examined by multiplex PCR using EVT-1 and EVT-2 primers, and EVS-1 and EVC-2 primers, respectively, as described in Table 1 [30]. Briefly, 50 μl of overnight culture of STEC strains was added to 450 μl of TE buffer and the mixture was boiled for 10 min and snap cooled on ice. After centrifugation at 10,000 *g* for 10 min, supernatant was collected and stored at –30°C for further use as boiled template. PCR was carried out in 20 μl of reaction mixture for each tube containing 1 μl of DNA template, 1 × PCR buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, and 1.5 mM $MgCl_2$), 0.2 mM of dNTP, 0.625 U of rTaq polymerase (Takara Bio Inc.) by using GeneAmp PCR system 2400 (Perkin-Elmer, Wellesley, MA, U.S.A.). PCR primer and conditions are described in Table 1. The PCR products were subjected to 2.0% agarose gel electrophoresis in TAE (40 mM Tris-acetate [pH 8.0], 1 mM EDTA) buffer followed by staining in ethidium bromide solution (2 $\mu g/ml$) and destaining in distilled water for 5–10 min each. Images were captured by Gel-Doc 2000 (Bio-Rad Laboratories).

PCR-RFLP: PCR was performed in a GeneAmp PCR System 2400 (Perkin-Elmer) using primer sets targeted to the region V or upstream region of *stx* genes in Stx-phage as shown in Fig. 1 and Table 1. The PCR products were analyzed by 0.4% agarose gel electrophoresis using HGT agarose and/or by field inversion gel electrophoresis using 1.0% pulsed-field certified agarose gel in 0.5 × TBE (45 mM Tris-borate [pH 8.0], 1.0 mM EDTA) buffer for 16 hr followed by staining with ethidium bromide (2 $\mu g/ml$). In these electrophoreses, a 1-kb or 2.5-kb DNA ladder (Takara Bio. Inc.) was used as the molecular mass standard. The run condition was generated by the autoalgorithm mode of CHEF Mapper pulsed-field gel electrophoresis (PFGE) system with a size range of 6 to 15 kb. The PCR products were further restric-

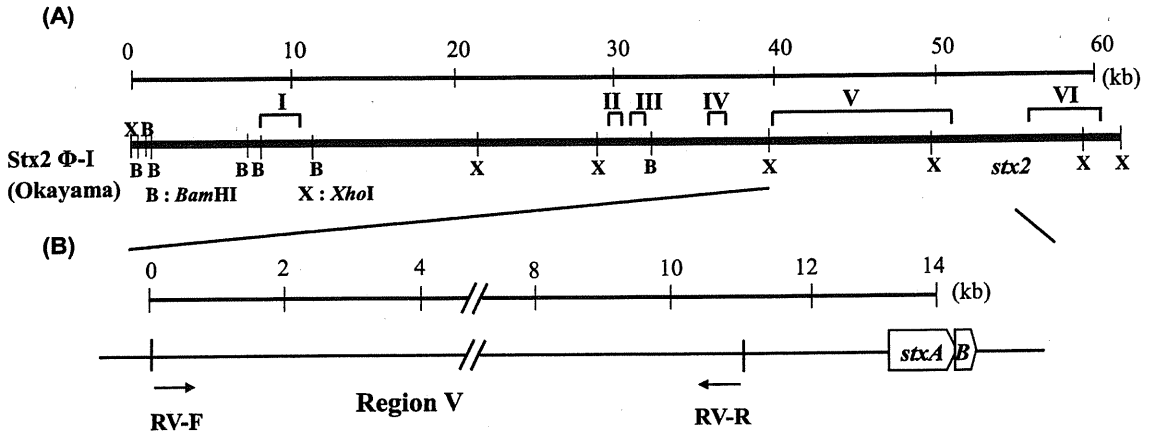

Fig. 1. (A) Restriction map of the Stx2ϕ-I genome and the location of six characteristic regions (I to VI). B and X represent *Bam*HI and *Xho*I, respectively. (B) Enlargement of the region V where PCR primer binding sites are located. Arrow indicates the location of primers, such as RV-F, and RV-R, respectively (Table 1).

Table 1. PCR primer and conditions used in this study

Primer	Sequence (5'–3')	Target	Aim	First denaturing	PCR conditions			Cycles	Amplicon	Reference
					Denaturing	Annealing	Extension			
EVT-1	CAACACTGGATGATCTCAG	*stx1*	Multiplex PCR	94°C 5 min	94°C 30 sec	55°C 30 sec	72°C 1 min	35	349 bp	[30]
EVT-2	CCCCCTCAACTGCTAATA									
EVS-1	ATCAGTCGTCACTCACTGGT	*stx2*		94°C 5 min	94°C 30 sec	55°C 30 sec	72°C 1 min	35	110 bp	[30]
EVC-2	CTGCTGTCACAGTGACAAA									
RV-F	GACATTGCTCCGTGTATTCACTCGTTGGAA	Region V	PCR-RFLP	94°C 1 min	98°C 20 sec	68°C 10 min[a)]		30	Variable	[37]
RV-R	ATTTTGCATTTCCTTCGCGCTGGTTTAGCC									

a) Annealing and extension steps were performed at the same time.

tion digested for 2 hr either by 10 U of *Bgl*I or 8 U of *Eco*RV. The digested products were then analyzed by 1.5% agarose gel electrophoresis and/or by field inversion gel electrophoresis using 1.0% pulsed-field certified agarose gel in 0.5 × TBE buffer for 18 hr. A 1-kb DNA ladder and a 2.5-kb DNA ladder (Takara Bio. Inc.) were used as molecular mass standards. The run condition was generated by the autoalgorithm mode of CHEF Mapper PFGE system with a size range of 1 to 10 kb for the *Bgl*I digest or 1 to 6 kb for the *Eco*RV digest. A model 1000 Mini-Chiller (Bio-Rad Laboratories) was used to maintain the temperature of the buffer at 14°C. The gels were stained and destained by similar way as mentioned above. The photographs of the electrophoretic patterns of the digested DNA (PCR-RFLP) were captured and recorded digitally using gel documentation system (Gel-Doc 2000, Bio-Rad Laboratories).

Data analysis: The discriminatory power of the PCR-RFLP typing for different O serogroup was evaluated using the Simpson's index of diversity as described previously [12].

RESULTS

Detection of stx1 and stx2 genes by multiplex PCR: A total of 200 STEC strains including 100 of O157, 50 of O26, 10 of O111 and 40 of other serogroups were analyzed for the presence of *stx1* and *stx2* genes by the multiplex PCR [30]. In O157 serogroup, 5, 46 and 49 strains were found to be positive for *stx1*, *stx2* and both *stx1* and *stx2* genes whereas in O26 serogroup 48 and 2 strains were positive for *stx1* and both *stx1* and *stx2* genes, respectively. In case of O111, 6 were positive for *stx1* and 4 for both *stx1* and *stx2* genes, while in case of non-O26/O111/O157, 20, 14 and 6 were positive for *stx1*, *stx2* and both *stx1* and *stx2* genes, respectively (Table 2).

PCR-RFLP for Shiga toxin-producing E. coli: Boiled templates of 200 STEC strains including 100 of O157, 50 of O26, 10 of O111 and 40 of non-O26/O111/O157 were amplified by primer set RV-F and RV-R and one to three amplicons ranging from 6.0 to 15.5 kb in size were yielded from 95 of O157, 48 of O26, 5 of O111 and 19 of non-O26/O111/O157 strains (Figs. 2A and 3A, Table 2). Subsequently PCR products obtained from 167 STEC strains were digested with either *Eco*RV or *Bgl*I and the RFLP patterns were compared to each other among each category of serogroups. Figs. 2B and 3B show the representative RFLP patterns of *Eco*RV digest whose discrimination ability is higher than those of *Bgl*I digest. In the case of O157, *Eco*RV digest yielded 4 to 11 fragments ranging from 450 bp to 5.7 kb in size (Fig. 2B) and *Bgl*I digest yielded 2 to 9 fragments ranging from 500 bp to 9.6 kb in size (data not shown). Although RFLP patterns of O157–30 (lane 31) and O157–31 (lane 32) by *Eco*RV were identical, those were differentiated by *Bgl*I digest (data not shown). In the case of O26, *Eco*RV digest yielded 4 to 14 fragments ranging from 150 bp to 4.4 kb in size (Fig. 3B, Lanes 2–9) while in O111, 5 to 6 fragments ranging from 900 bp to 8.5 kb were obtained by *Eco*RV digest (Fig. 3B, Lanes 10–13). In the case of non-O26/O111/O157, 2 to 12 fragments ranging from 150 bp to 5.7 kb were obtained by *Eco*RV digest (Fig. 3B, Lanes 14–30). Based on the RFLP profiles, 95 O157, 48 O26, 5 O111 and 19 non-O26/O111/O157 strains were classified into 41, 8, 4 and 17 groups, respectively (Tables 2 and 3).

DISCUSSIONS

Molecular typing methods have played important roles to trace the route of infection and identify the strain associated with outbreak, in particular diffuse outbreak [40]. Among them, PFGE is currently the most widely used molecular subtyping method for detecting outbreaks of *E. coli* O157:H7 because of its high resolution and reproducibility. However, there are several disadvantages and limitations in PFGE as described above. Therefore, the use of PCR-based method such as PCR-RFLP may be an alternative because of several benefits. For example, the PCR-RFLP (1) does not require special equipment, although FIGE was used in this study, a simple mini gel electrophoresis unit such as MUPID (Advance Co., Ltd., Tokyo) can be applicable, (2) can analyze large number of strains at the same time, (3) can complete rapidly within a day, (4) can analyze the RFLP without isolation of the strain [34], (5) can avoid smearing due to free radical produced during electrophoresis [37], and (6) is not affected by repeated subculture of a single isolate *in vitro* and passage through bovine or human gastrointestinal tract [35, 36]. In addition, (7) it is not necessary to send STEC strains but genomic DNA is enough in order to compare the RFLP patterns at two different laboratories, which is safer and easier to ship. For this reason, we have developed a simple, rapid and easy molecular subtyping method such as PCR-RFLP assay on the basis of genetic diversity of region V in Stx-phage and have shown its various utilities

Table 2. Subtyping of STEC strains by PCR-RFLP

Serogroup	PCR-RFLP typing pattern[a)]	Serotype	No. of isolates analyzed	*Stx* 1	*Stx* 2	PCR amplicons (kb)
	157–1	O157:H7	13	+	+	8.2/10.5
	157–2	O157:H7	10	–	+	11.6
	157–3	O157:H7	5	–	+	13.1
	157–3	O157	1	–	+	13.1
	157–4	O157:H7	5	–	+	11.6
	157–4	O157:H7	1	+	+	11.6
	157–5	O157:H7	6	+	+	8.2/13.1
	157–6	O157:H7	4	–	+	11.6/12.7
	157–7	O157:H7	4	+	+	11.7/14.3
	157–8	O157:H7	2	+	+	8.2/13.1
	157–8	O157:H–	1	+	+	8.2/13.1
	157–9	O157:H7	2	+	–	8.2
	157–10	O157:H7	2	–	+	11.4
	157–10	O157:H–	1	–	+	11.4
	157–11	O157:H7	2	+	+	8.2/10.3/10.5
	157–12	O157:H7	2	+	+	8.2/11.1
	157–13	O157:H7	1	+	+	8.2/13.2
	157–13	O157:H–	1	+	+	8.2/13.2
	157–14	O157:H7	1	+	+	11.4/13.2
	157–14	O157:H–	1	+	+	11.4/13.2
	157–15	O157:H7	2	+	+	8.2/12.2
	157–16	O157:H7	2	–	+	10.5
	157–17	O157:H7	1	+	+	10.6
	157–17	O157:H–	1	+	+	10.6
O157	157–18	O157:H7	1	–	+	11.5/11.8
	157–19	O157:H–	1	–	+	12.3/12.6
	157–20	O157:H7	1	–	+	9.5/10.0
	157–21	O157:H7	1	–	+	10.3/10.6
	157–22	O157:H7	1	–	+	12.3/12.8
	157–23	O157:H7	1	+	+	10.2/10.5
	157–24	O157:H7	1	+	+	11.7/15.5
	157–25	O157:H7	1	+	+	10.2/14.3
	157–26	O157:H7	1	+	+	10.5/11.1
	157–27	O157:H7	1	+	+	11.4/11.6
	157–28	O157:H7	1	+	+	8.2/9.8
	157–29	O157:H–	1	–	+	11.6/13.5
	157–30	O157:H7	1	+	–	11.8
	157–31	O157:H7	1	+	–	11.7
	157–32	O157:H7	1	+	–	10.1
	157–33	O157:H–	1	+	+	11.7
	157–34	O157:H7	1	–	+	12.5
	157–35	O157:H7	1	–	+	11.9
	157–36	O157:H7	1	–	+	13.1
	157–37	O157:H7	1	–	+	13.0
	157–38	O157	1	–	+	12.5
	157–39	O157:H–	1	–	+	13.0
	157–40	O157:H–	1	+	+	11.1
	157–41	O157:H7	1	+	+	12.2
	None	O157:H–	1	+	+	NA
	None	O157:H7	4	–	+	NA
	26–1	O26:H11	22	+	–	9.1/9.6/10.7
O26	26–1	O26:H–	7	+	–	9.1/9.6/10.7
	26–2	O26:H11	5	+	–	9.6/10.7
O26	26–2	O26:H-	2	+	–	9.6/10.7
	26–3	O26:H11	6	+	–	9.6/11.3
	26–3	O26:H11	1	+	+	9.6/11.3
	26–4	O26:H11	1	+	+	9.1/9.6/11.3
	26–5	O26:H11	1	+	–	8.0/9.6/12.0
	26–6	O26:H11	1	+	–	9.1/9.6/12.0
	26–7	O26:H–	1	+	–	9.1/9.6
	26–8	O26:H11	1	+	–	9.6
	None	O26:H11	2	+	–	NA
O111	111–1	O111:H–	2	+	+	8.7/11.3
	111–2	O111:H-	1	+	+	11.6
	111–3	O111:H–	1	+	–	13.2/13.7
	111–4	O111:H–	1	+	+	13.7
	None	O111:H–	5	+	–	NA
	38	O38:H21	1	–	+	9.2
	63	O63:H33	1	–	+	9.7
	80	O80:H–	1	–	+	6.0
	84–1	O84:H–	1	+	–	13.2
	84–2	O84:H–	1	+	–	10.0/13.3
	103–1	O103:H2	1	+	–	7.1
	103–1	O103:H3	1	+	–	7.1
	103–2	O103:H2	1	+	–	9.6
	121	O121:H19	2	–	+	7.4
	128	O128:H2	1	–	+	7.2/10.9
	145	O145:H–	1	+	–	10.9
	153	O153:H19	1	+	–	8.4/10.9
	165	O165:H–	1	+	+	12.3
	169	O169:H–	1	+	–	10.5/11.7
	UT–1	OUT:H–	1	+	–	11.7
	UT–2	OUT:H–	1	+	–	8.0/10.8
	UT–3	OUT:H–	1	+	–	9.6
	UT–4	OUT:H–	1	–	+	13.1
	None	O81:H31	1	+	–	NA
others	None	O18:H49	1	+	+	NA
	None	O48:H21	1	+	+	NA
	None	O153:HUT	1	+	+	NA
	None	OUT:H21	1	+	+	NA
	None	OUT:H36	1	+	+	NA
	None	O7:H–	1	+	–	NA
	None	O41:H14	1	+	–	NA
	None	O91:H14	1	+	–	NA
	None	O91:H21	1	+	–	NA
	None	O115:H10	2	+	–	NA
	None	O145:H–	1	+	–	NA
	None	O169:H19	1	+	–	NA
	None	O8:H19	1	–	+	NA
	None	O28ac:H–	1	–	+	NA
	None	O113:H–	1	–	+	NA
	None	O165:H19	1	–	+	NA
	None	O174:H21	1	–	+	NA
	None	OUT:H2	1	–	+	NA
	None	OUT:H7	1	–	+	NA

NA: not applicabl, UT: untypable.
a) One strain of 26–8 and one strain of 103–2 shows the same PCR-RFLP typing pattern.

and usefulness [34–37]. Shima *et al.* [37] initially evaluated the PCR-RFLP assay by using 204 STEC strains from our laboratory collections. The data showed that 202 STEC strains out of 204 yielding PCR amplicons were classified into 24 groups. PFGE patterns of representative STEC O157 strains also divided into 24 groups and, hence, were

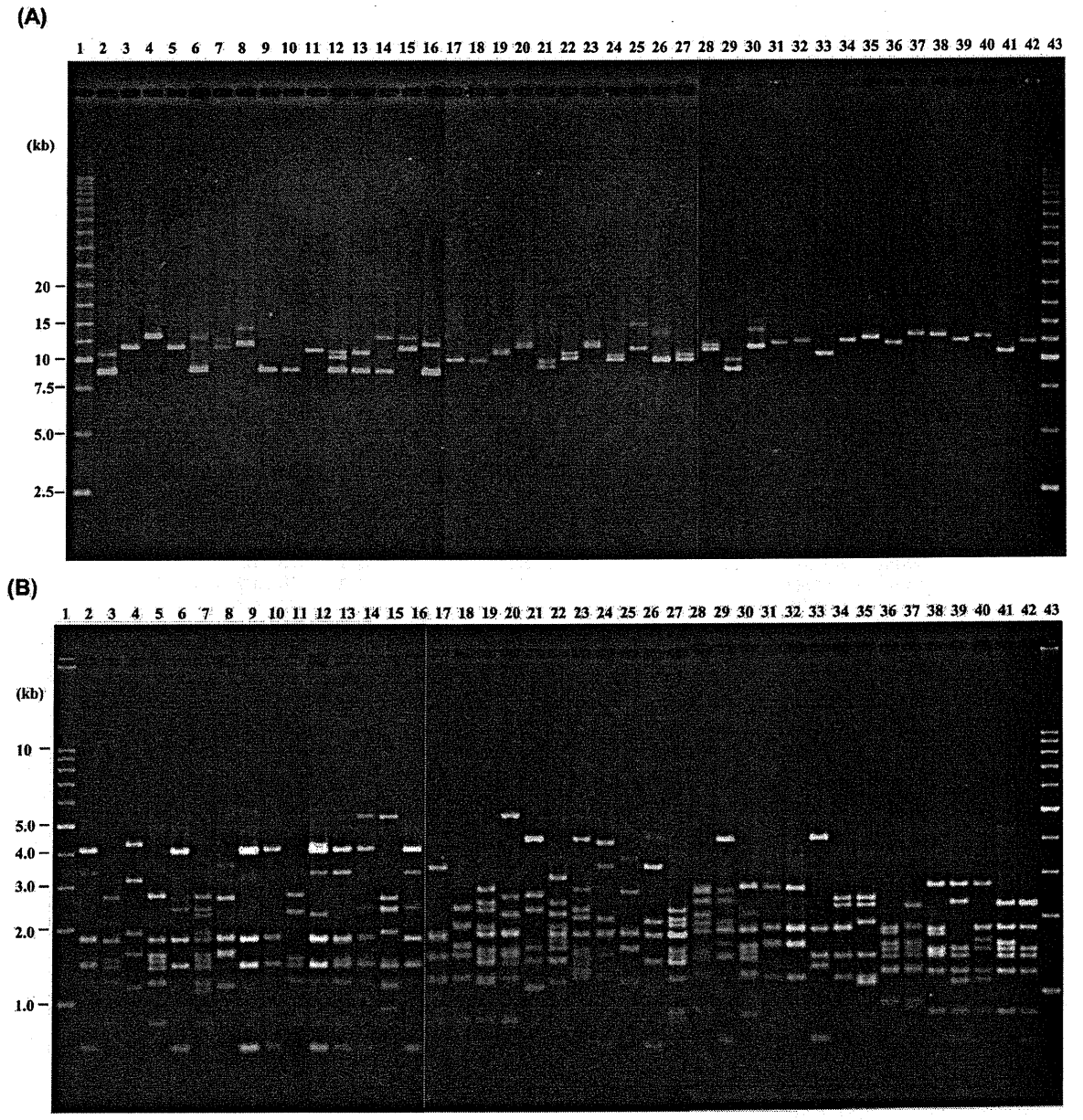

Fig. 2. (A) Field inversion gel electrophoresis of LA-PCR products obtained from 41 representative STEC O157 strains differentiated by the PCR-RFLP assay. Lanes: 1 and 43, 2.5-kb DNA ladder; 2, 157–1; 3, 157–2; 4, O157–3; 5, O157–4; 6, O157–5; 7, O157–6; 8, O157–7; 9, O157–8; 10, O157–9; 11, O157–10; 12, O157–11; 13, O157–12; 14, O157–13; 15, O157–14; 16, O157–15; 17, O157–16; 18, O157–17; 19, O157–18; 20, O157–19; 21, O157–20; 22, O157–21; 23, O157–22; 24, O157–23; 25, O157–24; 26, O157–25; 27, O157–26; 28, O157–27; 29, O157–28; 30, O157–29; 31, O157–30; 32, O157–31; 33, O157–32; 34, O157–33; 35, O157–34; 36, O157–35; 37, O157–36; 38, O157–37; 39, O157–38; 40, O157–39; 41, O157–40; 42, O157–41. (B) Field inversion gel electrophoresis of *Eco*RV digest of LA-PCR products obtained from 41 representative STEC O157 strains differentiated by the PCR-RFLP assay. Lanes: 1 and 43, 1-kb DNA ladder; 2, 157–1; 3, 157–2; 4, O157–3; 5, O157–4; 6, O157–5; 7, O157–6; 8, O157–7; 9, O157–8; 10, O157–9; 11, O157–10; 12, O157–11; 13, O157–12; 14, O157–13; 15, O157–14; 16, O157–15; 17, O157–16; 18, O157–17; 19, O157–18; 20, O157–19; 21, O157–20; 22, O157–21; 23, O157–22; 24, O157–23; 25, O157–24; 26, O157–25; 27, O157–26; 28, O157–27; 29, O157–28; 30, O157–29; 31, O157–30; 32, O157–31; 33, O157–32; 34, O157–33; 35, O157–34; 36, O157–35; 37, O157–36; 38, O157–37; 39, O157–38; 40, O157–39; 41, O157–40; 42, O157–41.

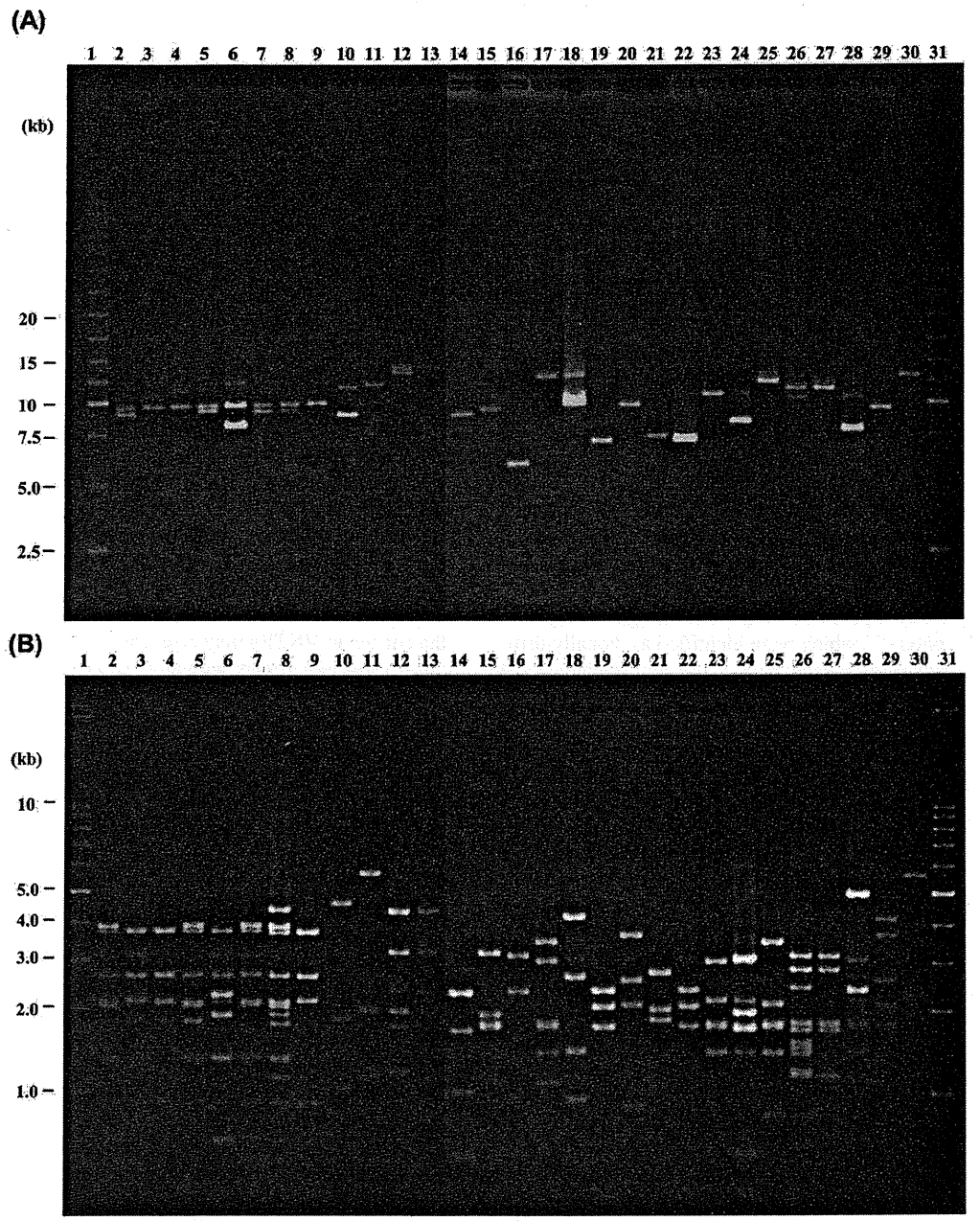

Fig. 3. (A) Field inversion gel electrophoresis of LA-PCR products obtained from 29 representative STEC non-O157 strains differentiated by the PCR-RFLP assay. Lanes: 1 and 31, 2.5-kb DNA ladder; 2, 26–1; 3, 26–2; 4, 26–3; 5, 26–4; 6, 26–5; 7, 26–6; 8, 26–7; 9, 26–8; 10, 111–1; 11, 111–2; 12, 111–3; 13, 111–4; 14, 38; 15, 63; 16, 80; 17, 84–1; 18, 84–2; 19, 103–1; 20, 103–2; 21, 121; 22, 128; 23, 145; 24, 153; 25, 165; 26, 169; 27, UT-1; 28, UT-2; 29, UT-3; 30, UT-4. (B) Field inversion gel electrophoresis of *Eco*RV digest of LA-PCR products obtained from 29 representative STEC non-O157 strains differentiated by the PCR-RFLP assay. Lanes: 1 and 31, 1-kb DNA ladder; 2, 26–1; 3, 26–2; 4, 26–3; 5, 26–4; 6, 26–5; 7, 26–6; 8, 26–7; 9, 26–8; 10, 111–1; 11, 111–2; 12, 111–3; 13, 111–4; 14, 38; 15, 63; 16, 80; 17, 84–1; 18, 84–2; 19, 103–1; 20, 103–2; 21, 121; 22, 128; 23, 145; 24, 153; 25, 165; 26, 169; 27, UT-1; 28, UT-2; 29, UT-3; 30, UT-4.

Table 3. Positive rate of LA-PCR and Simpson's Index of PCR-RFLP in 4 serogroup categories of STEC

Serogroup	Positive rate of LA-PCR (no. of strains/total strains)	Simpson's Index of PCR-RFLP[a] (no. of classifications /no. of strains)
O157	95% (95/100)	0.957 (41/95)
O26	96% (48/50)	0.603 (8/48)
O111	50% (5/10)	0.900 (4/5)
Others	48% (19/40)	0.988 (17/19)

Since the PFGE pattern of all the strains used is different, the Simpson's index of PFGE is equivalent to 1.00.
a) Simpson's Index of PCR-RFLP was calculated for the strains that produced amplicon by LA-PCR.

well correlated with those of PCR-RFLP. However, that study might be biased because only O157 serogroup strains were used and most of them were isolated at the same time period and in the close geographic area i.e., during large outbreaks occurred in 1996 in Sakai, Japan. Therefore, to properly evaluate the PCR-RFLP assay, defined STEC strains of different serogroups isolated at different time frame and from distal geographic area must be used in comparison to PFGE.

In this study, 200 STEC strains including not only O157 but also non-O157 which were identified as clonally different were randomly selected from 1,795 strains isolated at 42 regional public health institutes in Japan. Although out of 100 of O157 and 50 of O26 STEC strains, 95 (95%) and 48 (96%) strains yielded PCR amplicons, respectively, by the LA-PCR, only 5 (50%) and 19 (48%) strains yielded PCR amplicons out of 10 O111 and 40 non-O26/O111/O157 strains, respectively (Table 3). Ninety-five O157 strains yielding PCR amplicons were further classified into 41 groups by the restriction digestion. However, PCR products obtained from 48 of O26 strains were classified into only 8 groups. Five of O111 and 19 non-O26/O111/O157 strains were classified into 4 and 17 groups, respectively. Analysis of the typed strain by Simpson's Index indicated 0.957 of O157, 0.603 of O26, 0.900 of O111 and 0.988 of non-O26/O111/O157 (Table 3), respectively. However, positive rate of O111 and non-O26/O111/O157 strains was less than 50%. Taken together, these data indicate that the PCR-RFLP may be useful for the molecular subtyping of O157 but not for non-O157 STEC strains (Table 3).

Apart from PFGE, sequenced-based methods, such as multilocus sequence typing (MLST), have been used as powerful subtyping tools in molecular epidemiology [42]. These methods have several advantages of being easily standardized and automated, shorter assay times and totally comparable and transferable data between laboratories [19, 20, 24]. MLST analyzes the internal fragments of housekeeping genes to ascertain genetic relatedness among isolates. Although this method was successful for the differentiation of other organisms [9, 19, 46], it was unable to discriminate among *E. coli* O157:H7 isolates [28]. Alternatively, multiple-locus variable-number of tandem repeats analysis (MLVA) has been developed and successfully utilized for *E. coli* O157:H7 [14, 17, 28, 41]. In prokaryotic genomes, a wide array of repetitive DNA elements ranging from single to multiple repeats of nucleotides is present. MLVA is a PCR-based subtyping method that can be used to discriminate among isolates based on variable number of tandem repeats (VNTRs). In general, seven or more loci are selected and the choice of proper loci in MLVA is important for better subtyping. To obtain high discriminating result, selection of loci with high diversities is very crucial. However, more diverse loci may be more unstable and, therefore, the change in VNTRs may affect clonal turn over. Indeed a 5-day serial experiment with an *E. coli* O157 outbreak strain conducted by Noller *et al.* [27] showed a significant variation in at least one of the loci analyzed. Since VNTRs evolve so quickly, it is concerned that multiple MLVA types would emerge during an outbreak initially caused by a single clone.

PFGE alone is not sufficient and other typing methods may be necessary in epidemiological surveys [7, 10]. At this moment, MLVA would be the best but it would be better to combine the PFGE. Although MLVA can be automated and standardized, it requires DNA sequencer, which is very expensive and, hence, is not affordable by many laboratories or regional public health institutes. In any cases, each methodology has advantages and disadvantages. Therefore, the PCR-RFLP assay might be an alternative, which can be performed in an ordinal laboratory where PCR and electrophoresis are available and is not affected by clonal turn over *in vitro* and *in vivo* [35, 36].

In conclusion, the PCR-RFLP assay targeting region V is a practical method for molecular epidemiological studies especially for O157, the most important serogroup implicated in human diseases, although the discriminatory power of PCR-RFLP assay was little bit less than that of PFGE. Therefore, the PCR-RFLP assay may be useful for the initial survey before PFGE analysis or as a supplementary molecular subtyping in conjunction with PFGE while analyzing DNA fingerprints for O157:H7 STEC strains because of its simplicity, rapidity, easiness and reliability.

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