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

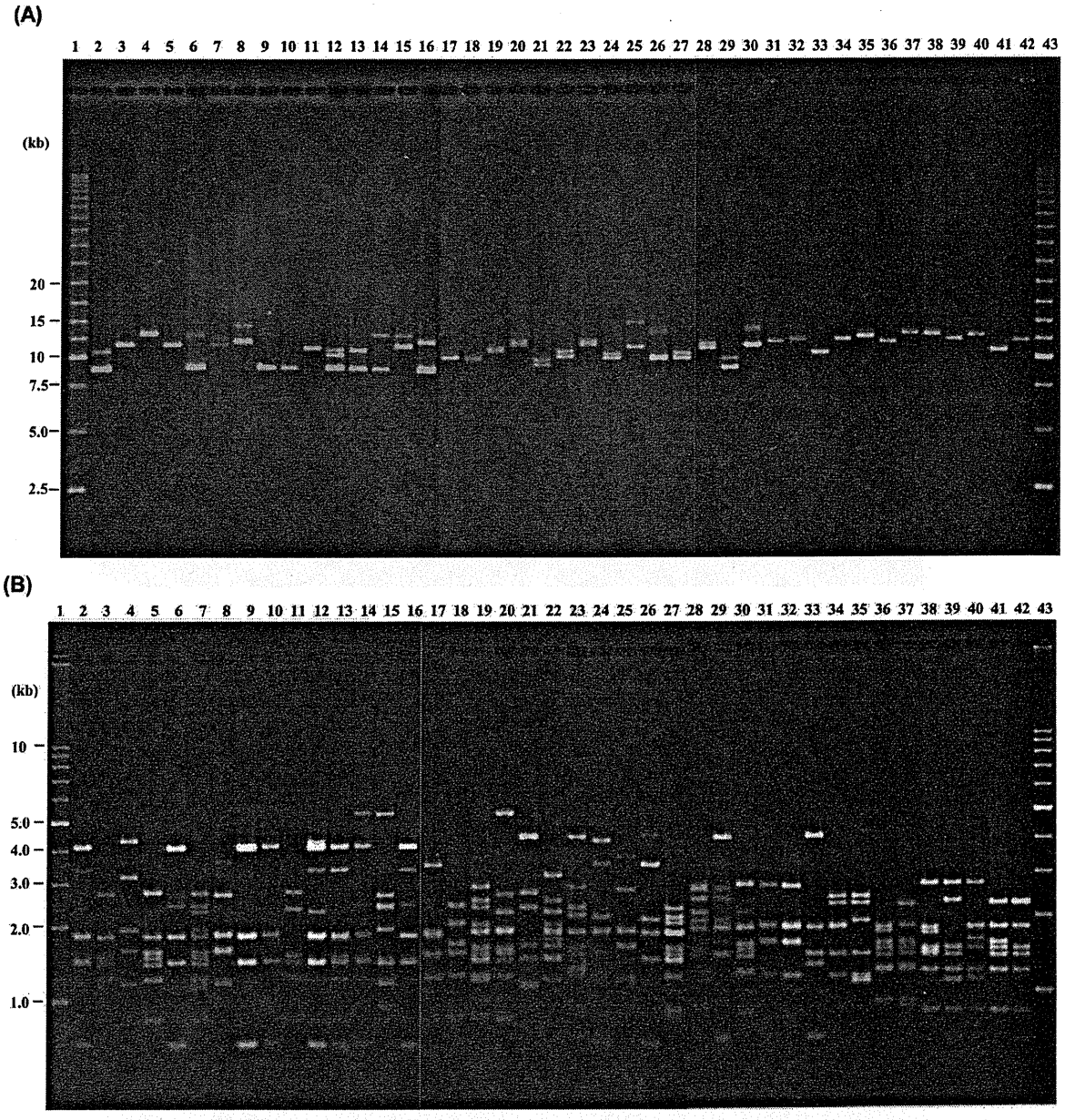

Fig. 2. (A) Field inversion gel electrophoresis of LA-PCR products obtained from 41 representative STEC O157 strains differentiated by the PCR-RFLP assay. Lanes: 1 and 43, 2.5-kb DNA ladder; 2, 157–1; 3, 157–2; 4, O157–3; 5, O157–4; 6, O157–5; 7, O157–6; 8, O157–7; 9, O157–8; 10, O157–9; 11, O157–10; 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.

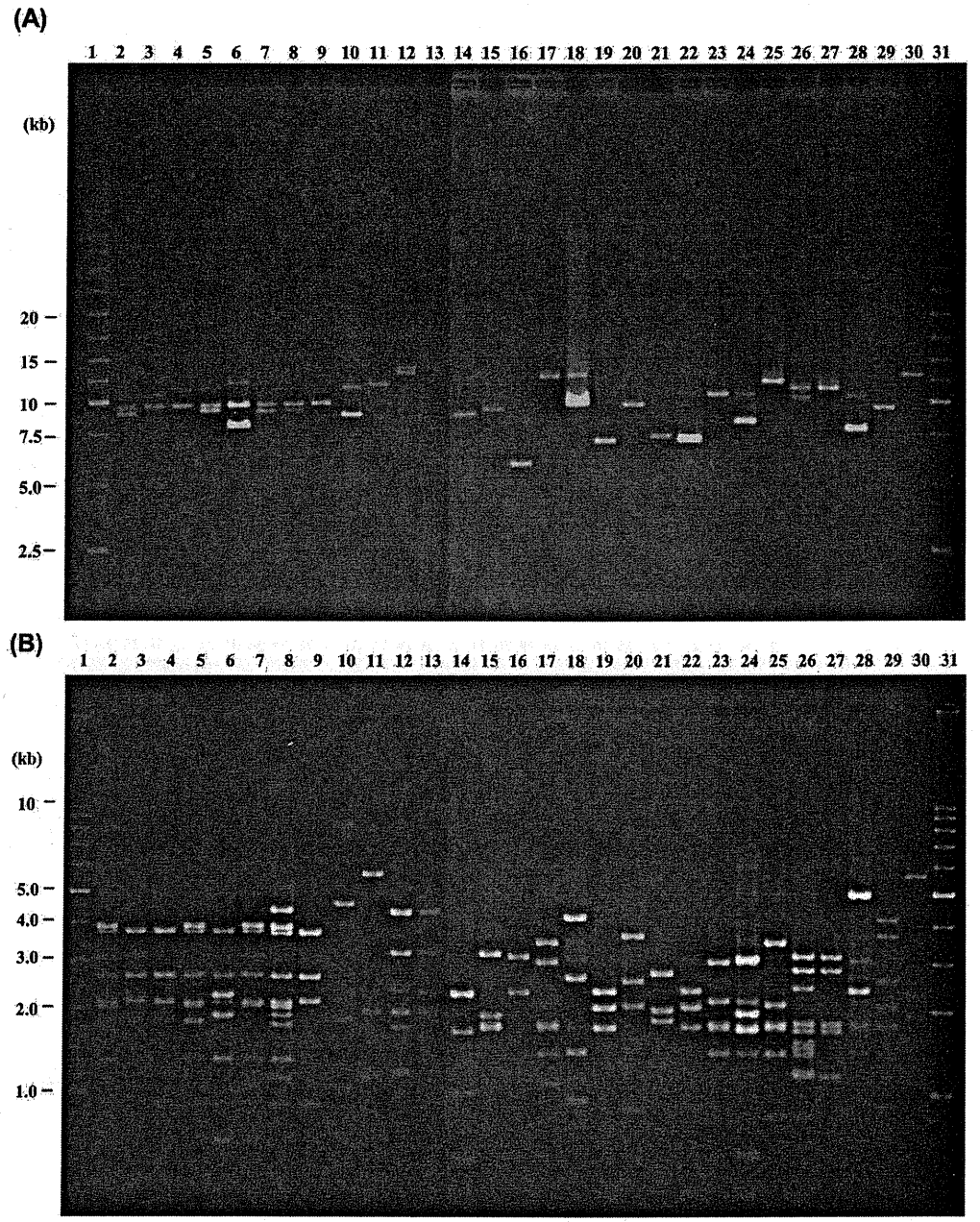

Fig. 3. (A) Field inversion gel electrophoresis of LA-PCR products obtained from 29 representative STEC non-O157 strains differentiated by the PCR-RFLP assay. Lanes: 1 and 31, 2.5-kb DNA ladder; 2, 26–1; 3, 26–2; 4, 26–3; 5, 26–4; 6, 26–5; 7, 26–6; 8, 26–7; 9, 26–8; 10, 111–1; 11, 111–2; 12, 111–3; 13, 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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