Molecular typing of Japanese *Escherichia coli* O157:H7 isolates from clinical specimens by multilocus variable-number tandem repeat analysis and PFGE

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The multilocus variable-number tandem repeat analysis (MLVA) method to target eight variable-number tandem repeat loci, based on agarose gel electrophoresis separation of multiplexed PCR products, and the PFGE method were applied to clinical isolates of *Escherichia coli* O157:H7 with the aim of comparing their performance as methods of typing this bacterium. Using MLVA, a total of 57 isolates from patients in Shizuoka prefecture, Japan, were divided into 20 types and classified into 23 PFGE types. Twenty-four isolates from four sporadic infections, four household contact infections and one outbreak that occurred in central parts of Shizuoka prefecture during August to November in 2005 were shown to be the same MLVA type, and most of the isolates had identical PFGE banding patterns, suggesting the diffuse outbreak in these parts of Japan. Thus, there was a good correlation between MLVA types and PFGE types, with both methods displaying broadly similar discriminatory powers. However, the MLVA typing proved to be a much easier and more rapid method for the analysis of *E. coli* O157:H7 strain relatedness to identify transmission routes. Hence, our MLVA method would be a suitable technique for routine typing in many laboratories, including public health agencies, and even in hospitals.

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INTRODUCTION

Escherichia coli O157:H7 has emerged as a food-borne pathogen causing severe illness with bloody diarrhoea and haemolytic uraemic syndrome (Besser *et al.*, 1999), and is of public health significance worldwide. Most *E. coli* O157:H7 infections are caused by exposure to bovine faeces-contaminated food or water, and by person-to-person transmission in household contact infections or outbreaks (Keen *et al.*, 2006; Parry & Salmon, 1998; Rangel *et al.*, 2005). In Japan, the extraordinarily large outbreaks of *E. coli* O157:H7 that occurred in 1990 and 1996 resulted in the infection of thousands of people and the death of seven patients by haemolytic uraemic syndrome (Izumiya *et al.*, 1997; Watanabe *et al.*, 1996). Since then, more than 2000 cases per year from sporadic infections, household

contact infections and outbreaks have been documented in Japan.

Molecular typing methods are increasingly used to assess the relatedness of *E. coli* O157:H7 strains, in particular to identify their transmission routes. PFGE-based typing is highly discriminative (Izumiya *et al.*, 1997; Swaminathan *et al.*, 2001), but it is time-consuming and cannot easily handle a large number of sample sets.

Multilocus variable-number tandem repeat analysis (MLVA) is a recently developed PCR-based technique (Lindstedt, 2005) used to target tandem repeats (TRs), which are areas of the bacterial genome that evolve rapidly (the definition of a variable-number TR (VNTR)) (van Belkum *et al.*, 1998). Targeting of these elements, which often vary in number among different strains of the same species, has been successfully used to discriminate between strains of prokaryotes such as *Bacillus anthracis* (Keim *et al.*, 2000), *Yersinia pestis* (Klevytska *et al.*, 2001), *Salmonella*

Abbreviations: MLVA, multilocus variable-number tandem repeat analysis; TR, tandem repeat; VNTR, variable-number tandem repeat.

enterica (Lindstedt *et al.*, 2003), *Neisseria meningitidis* (Yazdankhah *et al.*, 2005), *Mycobacterium tuberculosis* (Cowan *et al.*, 2002; Savine *et al.*, 2002) and *E. coli* O157:H7 (Noller *et al.*, 2003; Lindstedt *et al.*, 2004; Keys *et al.*, 2005). However, in *E. coli* O157:H7, MLVA-based typing requires sequencing to determine the number of short repeats (mostly 6 bp) at TR loci. Here, we describe a much simpler MLVA method based on the agarose gel electrophoresis separation of multiplexed PCR products for the analysis of *E. coli* O157:H7 strain relatedness, with the objective of identifying the transmission routes. Furthermore, we compared its performance with that of PFGE-based typing.

METHODS

***E. coli* O157:H7 isolates and culture.** A total of 57 *E. coli* O157:H7 isolates were obtained from 20 patients with sporadic infections, 6 from 1 outbreak and 31 from 11 household contact infections (2–5 patients per case) in Shizuoka prefecture, Japan, in 2005. Production of Stx was examined by the reversed passive latex agglutination test according to the manufacturer's instructions (Denka Seiken). Each isolate was cultured on a sorbitol MacConkey agar plate (Oxoid) for 18 to 24 h at 37 °C, and a single colony was transferred and spread onto a heart infusion agar plate and further incubated for 18 to 24 h at 37 °C.

MLVA. The entire *E. coli* O157:H7 EDL933 genome sequence (GenBank accession no. AE005174) was analysed to find the genetic loci of VNTR using Tandem Repeats Finder software (Benson, 1999). Of 112 VNTR loci detected in the genome, 19 were selected for designing PCR primers. From preliminary PCR experiments using these primers, we eventually chose eight VNTR loci as targets for MLVA typing (Table 1). Multiplexed PCR was performed using primer-pair sets for four combinations of two VNTR loci (VR1 and VR7, VR2 and VR5, VR3 and VR8, and VR4 and VR6). PCR reaction mixtures (20 µl) contained template DNA, 0.5 µM each primer, 2.5 mM dNTPs, 2.0 µl 10 × PCR buffer, and 1 unit *Ex Taq* hot start version (Takara-Bio). The mixture tubes were placed in a thermal cycler (GeneAmp PCR system 9700; Applied Biosystems) and the temperature was raised to 94 °C for 5 min, followed by 30 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s. The final hold was for 5 min at 72 °C. After PCR, the amplicons were electrophoresed in a 20 cm long 3% agarose gel (MetaPhor agarose; Cambrex) in a

Multi-submerge agar 30 (Atto) apparatus at 8 V cm⁻¹ for 3 h at 15 °C. Two DNA size markers of 20 and 100 bp ladders were mixed, and used for the estimation of PCR product sizes. After electrophoresis, the gels were stained and visualized with 0.5 µg ethidium bromide ml⁻¹. Based on the PCR product sizes, the number of repetitive DNAs at each VNTR locus was estimated, and the *E. coli* O157:H7 clinical isolates were classified based on the variation of the number of repeats at eight VNTR loci.

PFGE. PFGE analysis was performed according to the procedure described by Ribot *et al.* (2001). Briefly, 100 µl bacterial suspension, 20 mg proteinase K ml⁻¹ (Roche) and 110 µl 1% (w/v) SeaKem Gold agarose (Cambrex) kept at 60 °C were mixed. The solution was poured into the wells of a sample plug caster (Bio-Rad) and kept at 4 °C to solidify. The plugs were incubated with 5 ml lysis buffer (50 mM EDTA pH 8.0, 50 mM Tris, 1% *N*-lauroyl sarcosine and 0.2 mg proteinase K ml⁻¹) at 54 °C for 60 min, washed with distilled water at 54 °C for 15 min and washed three times with TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0) at 54 °C for 10 to 60 min. Then, the plugs were cut and treated with *Xba*I restriction enzyme (30 units per sample) at 37 °C for 2 to 3 h. Electrophoresis was performed in a 1% SeaKem Gold agarose gel with 0.5 × TBE (50 mM Tris, 45 mM boric acid, 0.5 mM EDTA) using the CHEF-DR III system (Bio-Rad) at pulse time of 2.2–54.2 s and 6 V cm⁻¹ for 20 h at 12 °C. After electrophoresis, the gel was stained with ethidium bromide solution and photographed under a transilluminator. Computer-assisted analysis for the comparison of PFGE banding patterns was performed with FingerPrinting II software (Bio-Rad) using the Dice coefficient according to the manufacturer's instructions. A dendrogram was constructed by UPGMA, and a tolerance of 1% in the band position was applied to the clustering.

RESULTS AND DISCUSSION

A total of 57 *E. coli* O157:H7 clinical isolates obtained from patients in Shizuoka prefecture, Japan, were examined by MLVA to target the eight VNTR loci of the genome. Multiplexed PCR revealed the targeted amplicons at the eight loci in all isolates tested, except in two isolates of each at the VR1 and VR4 loci (Table 2). Although the repeat array size is a very short sequence of 6 bp at VR1, VR 3, VR4, VR5 and VR8 loci (Table 1), we could successfully distinguish the number of repetitive DNAs among the isolates on the agarose gel (Fig. 1). As shown in

Table 1. Selected eight VNTR loci and primers used for MLVA of *E. coli* O157:H7

Locus	Location in EDL933	Array in EDL933*	Forward primer (5'-3')	Reverse primer (5'-3')	Product size (bp)†
VR1	271423–271478	6 × 9	ATTATTGGCGGAGCTTTCTG	TCTGGCTGCAAATAAAGACG	120
VR2	2103875–2104054	30 × 6	TGATCCCAAGTTACGCCATT	GACGATGACAATCACCACCA	285
VR3	2932247–2932284	6 × 6	AGCATCATCACCACGATCAC	ACTCTTCGTTGTGCGCCATGT	108
VR4	3557714–3557778	6 × 10	AATCGAAATACATGAACATAAGAAAAA	CCAATTGAATCTACAGTGGTCTG	129
VR5	4850327–4850359	6 × 5	CTGCCGGAGTATCAGCTTG	GGAAAACTGATGCAGACTCG	122
VR6	5361515–5361685	18 × 9	ACGCGAAGCTAACAAGGAAA	CCTTTACAACTCACCTGCGAAT	258
VR7	5454381–5454568	24 × 7	AACCCGGAATGGAAAATCTC	CAATGTGAGAGCGAAAAATGTC	278
VR8	5456065–5456102	6 × 6	GCTGCAGTTGCTCGGTTTTA	GAGTTTGACGATGGCGATCTA	145

*Repeat array size (bp) × no. of repeats.

†PCR product size based on *E. coli* O157:H7 EDL933.

Table 2. MLVA typing of 57 Japanese *E. coli* O157:H7 clinical isolates

MLVA type	No. of isolates	No. of repeats for VNTR locus:							
		VR1	VR2	VR3	VR4	VR5	VR6	VR7	VR8
A	1	4	6	8	NP	13	7	7	7
B	1	4	7	8	NP	8	7	8	3
C	2	6	4	5	6	6	6	5	3
D	3	8	4	10	10	16	7	4	5
E	1	8	6	4	17	5	9	7	8
F	2	8	6	6	9	6	9	7	7
G	1	8	6	6	10	6	9	7	7
H	1	8	6	6	11	6	9	7	7
I	1	8	6	7	11	8	7	4	6
J	1	9	6	6	11	6	9	7	7
K	1	9	6	8	10	6	9	7	8
L	1	10	6	5	6	5	8	5	8
M	1	10	6	6	15	5	9	7	6
N	24	12	6	4	16	5	9	7	8
O	7	13	6	6	11	5	8	7	8
P	1	14	6	4	17	5	9	7	8
Q	2	14	6	6	10	5	8	7	8
R	2	14	6	6	12	5	8	7	8
S	2	14	6	6	11	5	8	7	8
T	2	NP	6	7	6	10	6	3	3

NP, No product from PCR.

Table 2, the 57 isolates were divided into eight types with 4 to 14 repeats at VR1, three types with 4 to 7 repeats at VR2, six types with 4 to 10 repeats at VR3, eight types with 6 to 17 repeats at VR4, six types with 5 to 16 repeats at VR5, four types with 6 to 9 repeats at VR6, four types with 4 to 8 repeats at VR7, and five types with 3 to 8 repeats at VR8. Eventually, all isolates tested could be classified into 20

Fig. 1. Representative agarose gel MLVA profiles of Japanese *E. coli* O157:H7 clinical isolates. Lanes: M, DNA size markers (20+100 bp ladders); 1, S05-V9 (MLVA type C); 2, S05-V19 (MLVA type D); 3, S05-V42 (MLVA type L); 4, S05-V33 (MLVA type N).

types based on the variation of the number of repetitive DNAs at the 8 loci (MLVA types A to T in Table 2). Twenty-four isolates from four sporadic infections, four household contact infections (related cases 8, 9, 10 and 11 in Fig. 2) and one outbreak were shown to be the same MLVA type N, suggesting the predominance of *E. coli* O157:H7 during 2005 in Shizuoka prefecture of Japan.

The strain relatedness of 57 Japanese *E. coli* O157:H7 isolates was further analysed by PFGE typing (Fig. 2). A total of 19–24 DNA bands of the 57 isolates separated by PFGE were detected in the 30 to 500 kb range. By cluster analysis with 1% tolerance in band positions, isolates with identical PFGE banding patterns were considered as 'identical PFGE types'. We also considered any isolate with more than three different (in size or in number) bands in comparison with any other isolates as a 'unique PFGE type', and isolates with one or two different bands in comparison with the other isolates as 'similar PFGE types'. As shown in Fig. 2, the tree branched into two major clusters: one (10 isolates) consisting of 8 STX2-producing and 2 STX1-STX2-producing isolates, and another (47 isolates) composed of 46 STX1-STX2-producing and 1 STX2-producing isolates. By PFGE, the total 57 clinical isolates were classified into 23 types, and the isolates with identical PFGE banding patterns were further grouped into 8 identical PFGE types (indicated by the brackets on the right of the PFGE photos in Fig. 2). Twenty-two isolates from three sporadic infections, four household contact infections (related cases 8, 9, 10 and 11 in Fig. 2) and one outbreak, which had identical PFGE banding patterns, were considered as the major identical PFGE types. This corresponds well to the results obtained by the MLVA method (MLVA type N), except for two isolates (SV05-V25 and SV05-V30). Two to five isolates from each of the eleven household contact infections (related cases 1 to 11), except for S05-V25, were genetically related by the PFGE method.

The PFGE profiles were well correlated with the results of MLVA as follows: (i) 41 out of 46 isolates with MLVA types C, D, F, N, O, Q, R, S and T had PFGE banding patterns identical to those of other isolates with the same MLVA types (indicated by the brackets on the right of the PFGE photos in Fig. 2), except for S05-V31, S05-V24, S05-V13, S05-V25 and S05-V30; (ii) 8 out of 11 isolates with 'unique MLVA types' (MLVA types marked with asterisks in Fig. 2), except for S05-V47, S05-V4 and S05-V12, from sporadic infections had unique PFGE banding patterns (isolate with asterisks in Fig. 2); (iii) 3 isolates, S05-V31, S05-V13 and S05-V25, had only 1 or 2 different bands on PFGE from those of other isolates with MLVA types D, R and N, respectively (Fig. 2); (iv) 1 isolate with MLVA type R, S05-V24, was different only in the number of repeats at VR4 locus from those of 2 other isolates with MLVA type Q, although all 3 isolates in the related case 6 had identical PFGE banding patterns (Table 2, Fig. 2); (v) 3 isolates of S05-V47 (MLVA type G), S05-V4 (MLVA type J) and S05-V12 (MLVA type P), had only 1 or 2 different bands on

Fig. 2. Dendrogram of Japanese *E. coli* O157:H7 clinical isolates constructed based on PFGE with *Xba*I and the corresponding MLVA types. Clinical isolates from each case of household contact infection (corresponding to 'related case') and one outbreak are enclosed with a line, and the others are from sporadic infections. Identical PFGE types which were considered by cluster analysis with 1 % tolerance in the band positions are indicated by brackets on the right of PFGE photos. Isolates which have banding patterns considered as unique PFGE and MLVA types are indicated by asterisks after the isolate and MLVA type, respectively. STX, Shiga toxin.

PFGE and only 1 or 2 different numbers of repeats at the VR4 locus from those of the isolates with MLVA types J, G and N, respectively (Table 2, Fig. 2). The only striking

discrepancy between the results of MLVA and PFGE was found in one isolate, S05-V30, which had a unique PFGE type with more than 5 different bands in comparison with

any other isolates, but it was grouped into MLVA type N consisting of 24 isolates. The identity between the types of *E. coli* O157:H7 isolates in MLVA and PFGE was 93.0% (53/57).

The MLVA method, which was developed recently, is useful for bacterial typing targeting VNTR loci with less than 100 bp TR sequences distributed throughout the genome. Because the number of repetitive DNAs with a repeat array size of 6 bp varies on the *E. coli* O157:H7 genome, the repeats have been frequently used as targets of MLVA (Keys *et al.*, 2005; Noller *et al.*, 2003). To date, the number of such short repeats at loci has been determined by sequencing (Keys *et al.*, 2005; Lindstedt *et al.*, 2004; Noller *et al.*, 2003). However, we could estimate the number of such short repetitive DNAs more easily on agarose gel using the MLVA method described in this study. An important aspect of this technique is that the PCR target length at five VNTR loci with a repeat array size of 6 bp (VR1, VR3, VR4, VR5 and VR8) should be less than 200 bp, because the small size differences of the 6 bp repeats between PCR products of more than 200 bp might not separate well on agarose gel.

Noller *et al.* (2003) reported that MLVA to target seven VNTR loci has a discriminatory power higher than that of PFGE, because one of seven VNTR loci (Noller's TR2) with a repeat array size of 6 bp at 3 559 120–3 559 225 bp of *E. coli* O157:H7 EDL933 strain has a large variation in the number of repeats among isolates. According to their report, Noller's TR2 locus has been shown to vary between 4 and 58 repeats among 58 strains. Lindstedt *et al.* (2004) and Keys *et al.* (2005) confirmed such a large variation of Noller's TR2 by their MLVA. Noller *et al.* (2006) further reported that the number of repeats at one of seven VNTRs (Noller's TR2) was highly changed in 35 of 534 colonies after one to ten times broth-culture-passages from an *E. coli* O157:H7 single strain, but the number of repetitive DNAs at other loci had changed only in 0 to 4 colonies. In this study, however, we did not target Noller's TR2 locus for our MLVA typing, because the variation of the number of repeats at that locus is too large for our typing. It is almost impossible to design PCR primers to obtain less than a 200 bp product at Noller's TR2 locus. Hence, the discriminatory power of PFGE seems to be slightly superior to that of our MLVA typing, but the results obtained by MLVA and PFGE in this study were well correlated. In both MLVA and PFGE, the *E. coli* O157:H7 clinical isolates from respective sporadic infections were mostly a unique type, and the isolates from household contact infections and from an outbreak were clustered in each case with some exceptions. A public health significance of this finding is that 24 isolates with MLVA type N were predominant (42.1%, 24/57) and 23 of those 24 isolates had identical or similar PFGE types. A total of 23 out of 24 isolates, not including S05-V30, were obtained from 8 cases of 3 sporadic infections, 4 household contact infections and 1 outbreak that occurred in central parts of Shizuoka prefecture during August to November in 2005, suggesting

that a diffuse outbreak might have occurred in these parts of Japan during that season.

In Japan, the National Institute of Infectious Diseases and prefectural public health institutes have made plans to set up a PFGE-based national network (PulseNet Japan) for monitoring the cross-prefectural spread of Japanese O157 strains (Matsumoto *et al.*, 2005). If the PulseNet national database eventually becomes available, the use of two or more endonucleases rather than a single enzyme in PFGE would be more useful for the precise classification of the strains as suggested by Gupta *et al.* (2004). Without the organization of the PulseNet surveillance system, the use of multiple enzymes will probably further complicate and confuse the interpretation of PFGE results.

As mentioned above, MLVA typing based on the agarose gel electrophoresis separation of multiplexed PCR products, as well as PFGE typing based on *Xba*I digestion, is available for the analysis of *E. coli* O157:H7 strain relatedness in order to identify transmission routes. Because our MLVA method is a much easier and more rapid technique, it will facilitate the typing and molecular epidemiology of *E. coli* O157:H7 clinical isolates in research institutes, public health agencies and even hospitals.

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