Method

Multiple-Locus Variable-Number Tandem Repeat Analysis Scheme for Non-O157 Shiga Toxin-Producing *Escherichia coli*: Focus on Serogroups O103, O121, O145, O165, and O91

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SUMMARY: Non-O157 Shiga toxin-producing *Escherichia coli* (STEC) infections are a growing concern for public health. The number of sporadic cases and outbreaks of non-O157 STEC infections have increased in recent years. Molecular subtyping is an essential tool that allows high-resolution and rapid differentiation of isolates, identification of case clusters, and detection of outbreak clusters. Multiple-locus variable-number tandem repeat analysis (MLVA) is one of the most useful typing methods for differentiating isolates that cause foodborne diseases. In Japan, serogroups O26, O111, O103, O121, O145, O165, and O91 have been frequently isolated or associated with severe cases of non-O157 STEC infections. In this study, we designed an MLVA scheme (MLVA43) for serogroups O103, O121, O145, O165, and O91 by adding 26 new loci to an MLVA scheme (MLVA17) previously developed by our group for serogroups O157, O26, and O111 using 17 loci. We found that the discriminatory power of MLVA43 was comparable to that of pulsed-field gel electrophoresis (PFGE) for serogroups O103, O145, O165, and O91, and superior to that of PFGE for O121. MLVA43 identified more profiles than did MLVA17, except for serogroup O111 with 707 isolates. The MLVA43 scheme will enable rapid detection of outbreak clusters, which will aid in implementing rapid control measures against non-O157 STEC infections.

INTRODUCTION

Shiga toxin-producing *Escherichia coli* (STEC) is an important etiological agent of food-borne diseases. The main symptoms of STEC infections are abdominal pain, watery diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome (HUS), with potentially fatal outcomes (1). More than 3,000 cases of STEC infections are annually reported in Japan (2). Though O157 is the most commonly isolated STEC serogroup, non-O157 STEC serogroups have been increasingly isolated from sporadic cases and outbreaks in recent years. Serogroups O26, O111, O103, O121, O45, and O145 have been isolated most frequently in the United States (3), whereas serogroups O26, O111, O103, O121, O145, O165, and O91 have been most frequently isolated or associated with severe cases in Japan (2).

Molecular subtyping of STEC is essential in cluster identification and outbreak investigation. The traditional “gold standard” method for molecular subtyping is

pulsed-field gel electrophoresis (PFGE) (4). However, PFGE is time-consuming and requires high level of technical skills; therefore, multiple-locus variable-number tandem repeat analysis (MLVA) using 17 loci has been introduced in Japan for molecular subtyping and surveillance of STEC serogroups O157, O26, and O111 (5,6). MLVA differentiates the isolates based on the difference in the number of tandem repeats of several variable-number tandem repeat (VNTR) loci located on the bacterial genome (7). The target loci are amplified by multiplex PCR, and the PCR products are separated and sized using capillary electrophoresis. An allele type is assigned to each VNTR locus, based on the copy number of repeats at each locus, which is an integer value. The string of allele types for several VNTR loci constitutes an MLVA type. MLVA is advantageous because the procedure is simple and rapid, and the data obtained are portable and discrete to enable comparison among multiple laboratories (8). MLVA has been applied for typing of bacteria that cause food- and water-borne diseases, including *Salmonella enterica* serotypes Typhimurium and Enteritidis, *Shigella sonnei*, and *Vibrio cholerae* (9–13).

The disadvantage of MLVA is that its discriminatory power can vary among different serogroups of the target organism. In our previous study, we developed an MLVA scheme using 17 loci for STEC serogroups O157, O26, and O111 (5). Recently, another MLVA scheme was reported for serogroups O26, O111, O103,

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O121, O45, and O145 (14). However, these schemes do not include all the serogroups of O103, O121, O145, O165, and O91 that pose serious threats to public health and/or are frequently isolated in Japan. Thus, the aim of this study was to design an MLVA scheme for non-O157 STEC serogroups isolated in Japan, particularly serogroups O103, O121, O145, O165, and O91.

MATERIALS AND METHODS

Bacterial strains: A total of 707 STEC clinical isolates were used in this study. They were isolated in Japan between 2007 and 2017, but most of them were isolated in 2017 ($n = 523$). A total of 184 isolates (isolated between 2007 and 2016) were used for optimization of test conditions of MLVA and for comparison of the discriminatory powers of MLVA and PFGE. All the isolates were associated with either outbreaks or sporadic cases, and one representative isolate was chosen from each outbreak. An additional set of 52 isolates (36 isolates from outbreak A, and 18 isolates from outbreak B; serogroup O103) were used in cluster analyses of outbreak-related isolates. They were analyzed together with two of the 707 isolates. Patient information was anonymized in this study.

MLVA: MLVA was performed as previously described by Izumiya et al. (5) with few modifications. Primer sets for the 17 loci from the previous study are shown in Table 1 (Mix R1 and R2). The genome sequences of the strains listed in Table 2 were searched for tandem repeats *in silico* and 26 additional loci were identified. Primers were designed using DNASIS Pro software (Hitachi Solutions, Ltd., Japan), and their sequence specificities were examined by performing a BLAST search against the NCBI nucleotide database. The primer pairs were designed to comply with the conditions of multiplex PCR. Primer sets for the 26 additional loci are shown in Table 1 (Mix q1 and q2). One primer from each pair was fluorescently labeled for ABI PRISM GeneScan Analysis (Thermo Fisher Scientific K.K., Tokyo Japan; <https://www.thermofisher.com/jp/ja/home/life-science/oligonucleotides-primers-probes-genes/applied-biosystems-custom-primers-probes.html>). Template DNA was prepared using DNeasy Blood & Tissue Kit (Qiagen K.K.-Japan, Tokyo, Japan), and quantified using NanoDrop spectrophotometer (Thermo Fisher Scientific K.K.) or by heat extraction of bacterial cells. The reaction mixture for PCR contained 1 μ L of template DNA (30–50 ng) in a total volume of 15–25 μ L. The concentration of DNA obtained by heat extraction was adjusted so that the resulting signals of the amplified products were comparable to those of DNA extracted using the kit. PCR was performed using QIAGEN Multiplex PCR Kit (Qiagen K.K.-Japan) and Platinum Multiplex PCR Master Mix (Thermo Fisher Scientific K.K.) following the manufacturer's instructions. The PCR conditions were as follows: initial denaturation at 95°C for 15 or 2 min (depending on the kit used); 35 cycles of denaturation at 95°C for 20 s, annealing at 60°C for 90 s, and extension at 72°C for 60 s; and final extension at 72°C for 10 min. The amplified PCR products were diluted and mixed with GeneScan 600LIZ size standard in Hi-Di Formamide (Thermo Fisher Scientific K.K.).

The diluted PCR products were sized using Applied Biosystems 3500xL Genetic Analyzer (Thermo Fisher Scientific K.K.).

PFGE: PFGE was performed for the 184 isolates according to the standardized PulseNet protocol using *Xba*I restriction enzyme (Roche Diagnostics GmbH, Mannheim, Germany) (15). PFGE patterns were analyzed using BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium). A dendrogram was generated based on the Dice coefficient and the unweighted pair group method with arithmetic means (UPGMA), with 1.2% positional tolerance. Isolates included in a single leaf of the dendrogram with over 95% similarity were regarded as a PFGE partition, and are referred to as “PFGE95” hereafter.

Analysis of MLVA data: Fragment data were evaluated and converted to repeat copy numbers using GeneMapper (Thermo Fisher Scientific K.K.) and GeneMarker (SoftGenetics LLC, PA, USA) software. Data were analyzed using BioNumerics software as previously described (8). Fragments with no PCR product were assigned a repeat copy number of -2 . Simpson's diversity index (D) and 95% confidence intervals (CI) were calculated according to the formula described previously (16). “Moderate” and “high” indicate D values greater than 0.15 and 0.5, respectively.

Whole genome sequencing: Complete genome sequences were obtained using MiSeq platform (Illumina, CA, USA). Genomic DNA libraries were prepared using Nextera XT DNA Sample Prep Kit (Illumina). The pooled libraries were subjected to multiplexed paired-end sequencing (300-mer $\times$ 2). Minimum coverage for the analyses was 40 $\times$. The short reads were assembled using SPAdes v. 3.11.1 with the “-careful” option (17).

Data availability: The assembled contigs used in this study were deposited in the DNA Data Bank of Japan. Accession numbers: BLJC01000000 for JNE080262, and BLJD01000000 for JNE160956.

RESULTS

Locus selection: We searched the complete and draft genome sequences of *E. coli* O26 ($n = 2$), O111 ($n = 2$), O103 ($n = 4$), O121 ($n = 1$), O145 ($n = 1$), O165 ($n = 1$), and O91 ($n = 2$) strains for tandem repeats (Table 2). Around 20 candidate loci were identified in each strain. The candidate loci were further screened to eliminate overlapping loci (Table 3). Using 184 isolates obtained between 2007 and 2016, 26 additional loci were obtained by performing two separate multiplex PCR amplifications (q1 and q2 in Table 1). Together with the reaction mixtures from the previous study, a total of 43 loci were obtained by performing four multiplex PCR amplifications (MLVA43; Table 1).

Comparison of discriminatory power between MLVA and PFGE: The 184 isolates included two O26, two O111, 57 O103, 63 O121, 17 O145, 23 O165, and 20 O91 isolates. MLVA43 and PFGE95 identified 129 and 116 types, respectively, and the D values were 0.991 and 0.971, respectively (Table 4). The discriminatory powers of the two methods were comparable among serogroups O103, O145, O165, and O91. The discriminatory power of MLVA43 for serogroup O121

MLVA Scheme for Non-O157 STEC

Table 1. Primers used in this study

Mix ¹⁾	Locus		Sequence (5'-3')	Conc. (μ M)	Dye	Alternative name ²⁾
R1	EH111-8	F	CCGGACGAGAGGGAGTAAATGAA	0.03	PET	
	EH111-8	F1	CCGGGCGAGTAGGAGTAAATGAA	0.03	PET	
	EH111-8	R	CATAAATTATGCTTAATGGAATTAGTAACGCTG	0.03		
	EH111-8	R1	CATGAATTATGCTTAATGGAATTAGTCAAGCTG	0.03		
	EH157-12	F	ACAGTACCCATGCCAGCAA	0.04	PET	
	EH157-12	R	GAAAGCTGGGTGAAAACACCGATGC	0.04		
	EHC-1	F	GTGCGTAACCTGCTGGCACA	0.04	VIC	SVL-3
	EHC-1	R	CGCGGCTGCCGGAGTATC	0.04		
	EHC-2	F	CCAGTTCGGCAGTGAGCTG	0.04	VIC	SVL-1
	EHC-2	R	ACGCTGGTCCGGGAGATTAT	0.04		
	EHC-5	F	ATACTACAGACGTCTGCTGATGA	0.04	NED	
	EHC-5	R	CCGCTTTGTTACCGGTCTTTTTC	0.04		
	O157-25	F	GCCGGAGGAGGGTGATGAGCGGTTATATTAGTG	0.04	PET	
	O157-25	R	GCGCTGAAAAGACATTCTCTGTTTGGTTTACAC	0.04		
	O157-3	F	GGCGGTAAGGACAACGGGGTGTGTTGAATTG	0.04	NED	
	O157-3	R	GAACAACCTAAAACCCGCCTCGCCATCG	0.04		
	O157-34	F	TGTTACCAACGCGAAGCTAACAAG	0.04	FAM	
	O157-34	R	AGGCATTAATAGCAGATGTTC	0.08		
	O157-9	F	GCGCTGGTTTAGCCATCGCCTTCTTCC	0.04	VIC	SVL-2
	O157-9	R1	TTCATTAATAAATAAATCCCATGGAAAATATTTTGTG	0.12		
	O157-9	R	GTGTCAGGTGAGCTACAGCCCGCTTACGCTC	0.08		
R2	EH111-11	F	GTCAGTAGTTGCGGCTGTAATATTGAAGA	0.04	FAM	
	EH111-11	R	CCTTGTGCATTGAGTTCTGTACATAG	0.04		
	EH111-14	F	ATGAAATTATCGCAGCATACAATCG	0.04	FAM	SVL-10
	EH111-14	R	GGGTTTCCATTTTCTTTACCTTCAGG	0.04		
	EH26-7	F	CCCCTATCAAACTGATACCCGATAAG	0.08	PET	
	EH26-7	R	CGCCGGAAGGCAAAAGATCAT	0.08		
	EHC-6	F	ATGGAGAACCGTCTGAGTGC	0.04	NED	SVL-12
	EHC-6	R	TCAGAAATCATCTCCCGGCTCAAC	0.04		
	O157-17	F	GCAGTTGCTCGGTTTAAACATTGCAGTGATGA	0.04	VIC	
	O157-17	R	AGAAATGGTTTACATGAGTTTGACGATGGCGATC	0.04		
	O157-19	F	GCAGTGATCATTATTAGCACCGCTTTCTGGATGTTT	0.04	NED	
	O157-19	R	CGGGCAGGGAATAAGGCCACCTGTAAAGC	0.04		
	O157-36	F	GGCGTCCTTCATCGGCCTGTCCGTAAAC	0.04	NED	
	O157-36	R	GCCGCTGAAAGCCCACACCATGC	0.04		
	O157-37	F	AATCAGAGCGGCAGGAAAAAGAAGA	0.04	PET	SVL-11
	O157-37	R	GGGCTTCTGTCTTTTCAGACCTG	0.04		
q1	q1701	F	TTCATAGTTCAGATCTCAATTTTTCG	0.04	FAM	
	q1701	R	CACGGCTAAACAGTAAACAGTTAATT	0.04		
	q1702	F	ACTGCATAGGCACTGATTAACATTC	0.04	VIC	
	q1702	R	GGCAAAAGTGTTTTCTCTTTTGT	0.04		
	q1705	F	ATACGCTGAATCGGCTTCC	0.04	NED	
	q1705	R	ACATTGATGTCACGACAGAAGATT	0.04		
	q1708	F	GCCAGACTATCACTATTGATCTGAAG	0.04	FAM	
	q1708	R	CTAGCATTAACAACAGCACCATCT	0.04		
	q1710	F	ATTGTCTGCATGTACACCAATTTT	0.04	VIC	
	q1710	R	ATACGTTGGCTGATAAGACGTTAAA	0.04		
	q1712	F	ATAGCACATCTGGTAGCTCCTACAC	0.04	PET	
	q1712	R	GAAAGATACCGTTGCAATAATGAAT	0.04		
	q1716	F	TAGTGCAACTACTTGTGGATAGTGC	0.04	VIC	

Mix ¹⁾	Locus		Sequence (5'-3')	Conc. (μ M)	Dye	Alternative name ²⁾
	q1716	R	GATCTACCTACCATTGTCATTGGAC	0.04		
	q1724	F	GTCGTCTGTGGGATGCTCAA	0.04	PET	SVL-5
	q1724	R	CAGCAATAACAGCAGGACGA	0.04		
	q1725	F	TTCTAAAGGTCTCAATATTGATTGAATGACG	0.08	PET	SVL-6
	q1725	R	TGCACCCTCCCATCGTTTCTGTTTCC	0.08		
	q1726	F	TATCGCATTTTATCGAACATAGTGA	0.04	PET	
	q1726	R	ATCCATGGAGAACACTTTATCTACC	0.04		
	q1727	F	GTAATTGACGACATCGGCTTTAATA	0.04	FAM	
	q1727	R	CTCGCTGGATGATTTTGAATTT	0.04		
	q1730	F	TTATTTGAACAGAATCTGGAACAGC	0.04	NED	
	q1730	R	GTAATGCAAAGGACAAAAGAAACAG	0.04		
	q1731	F	ATTCAGGTAGGTCATGCATAAAAT	0.04	NED	
	q1731	R	ATGGAGAAAATGTGATGCCTAGAG	0.04		
q2	q1704	F	CAGTGTATATCCATCTTCTGGTGAG	0.08	FAM	
	q1704	R	GTATTCAGGTTTCGGCTGTCTG	0.08		
	q1707	F	CGAGCATACTCTGTCTTGTATGAG	0.04	NED	
	q1707	R	TGAACATACAGGTGGACAACAATAC	0.04		
	q1711	F	ATATACGGCAGGTGGAGTCATC	0.04	FAM	
	q1711	R	CGGGGACGTGGTTAGTTTAC	0.04		
	q1714	F	GTAACAGTGGTAACGAAACGCTCT	0.04	VIC	
	q1714	R	CAGTTTTAACTGCTCATCATTGACA	0.04		
	q1715	F	TCTATGGTACAGAACCTGTTTTCAC	0.08	VIC	
	q1715	R	GTAAAGCATTGATTTGCCTTCC	0.08		
	q1717	F	CAACCTTGTTTGTTAAGGTTGAGAT	0.06	FAM	
	q1717	R	GGGATCTCTAATACTGCTGTACGTC	0.06		
	q1718	F	AGCAAAGTGAAGTATCTCAACAAG	0.04	VIC	
	q1718	R	AGTAACAGAGCTCGGTATCTCAATC	0.04		
	q1718	R1	CATCTACTCATAGGGGAATTTTGTTT	0.04		
	q1720	F	GCGAACAGCTGTATCTTAATGAGTT	0.04	PET	
	q1720	R	GAGGATTAAATTTTCCCAACTGTTT	0.04		
	q1721	F	TTGCAAAGACCATATCAGTGAGTAA	0.04	NED	
	q1721	R	GTTTCGGTAACAGCTTTATGCAG	0.04		
	q1722	F	ACTATCGTTTTAATAACCGGGATGA	0.06	PET	
	q1722	R	GAAAAAGGCAGTATAAAAACAGCAG	0.06		
	q1723	F	AAATCGGGCGGGAAGAAG	0.04	PET	SVL-23
	q1723	R	GGGCGTAAAAAGCAATAAAGG	0.04		
	q1728	F	ACTTAATCCCTTGAGGAACAGGAGT	0.04	NED	
	q1728	R	GGGAACTTGACTTCATACTGTGAT	0.04		
	q1729	F	GTTGTGCTGGGGTAAGAAATGAATA	0.04	FAM	
	q1729	R	G TTCAGTCAGAACGGGACAAGAAT	0.04		

¹⁾: multiplex combination.

²⁾: name in reference 14.

was superior to that of PFGE95.

For further evaluation of the selected loci, the experiment was expanded to a total of 707 isolates. The isolates included 272 O26, 38 O111, 133 O103, 123 O121, 50 O145, 31 O165, and 60 O91 isolates. MLVA17 and MLVA43 identified 396 and 468 types, respectively (D values: 0.993 and 0.997, respectively). The discriminatory powers of MLVA17 and MLVA43 are shown in Table 4, and the D value of each locus is shown in Table 5. The frequency of null alleles at each locus and the allelic ranges are shown in Table 6.

The characteristics of each serogroup or loci were as follows:

O26: The overall discriminatory power of MLVA43 was similar to that of MLVA17. MLVA43 identified more types than did MLVA17. Loci q1704, q1717, q1720, q1722, q1725, EHC-1, EHC-2, EHC-5, EHC-6, EH26-7, O157-9, and O157-37 exhibited moderate to high D values. Loci q1720 and q1722 were highly specific for serogroup O26 and exhibited low frequency of null alleles.

O111: The overall discriminatory power of MLVA43

Table 2. Strains used for sequence screening of VNTR loci

<i>Escherichia coli</i> serotype, strain	Accession No.	Reference ¹⁾
O26:H11 str. 11368	NC_013361.1	(19)
O26:H11 str. FWSEC0001	CP031922.1	na
O111:H- str. 11128	NC_013364.1	(19)
O111:H- str. JNE080262	BLJC01000000	This study
O103:H2 str. 12009	NC_013353.1	(19)
O103:H2 str. 2015C-3163	CP027219.1	(20)
O103:H25 str. 2013C-3264	CP027544.1	(20)
O103:H25 str. NIPH-11060424	AGSG00000000	(21)
O121:H19 str. FWSEC0006	CP031910.1	na
O145:H28 str. RM13514	CP006027.1	na
O165:H25 str. 2013C-4830	CP027325.1	(20)
O91:H- str. JNE160956	BLJD01000000	This study
O91:H14 str. 2013C-4404	CP027376.1	(20)

¹⁾: na, not available due to direct submission.

was the same as that of MLVA17. Loci q1702, q1721, q1725, EHC-1, EHC-2, EHC-5, EHC-6, EH111-11, EH111-8, O157-9, and O157-37 exhibited moderate to high D values. Variations at loci EH111-11, q1702, and q1721 were uniquely observed in serogroup O111. Loci EH111-11 and q1702 were ubiquitously amplified, but locus q1721 exhibited a high frequency of null alleles.

O103: The overall discriminatory power of MLVA43 was similar to that of MLVA17. MLVA43 identified more types than did MLVA17. Loci EHC-2, EH111-14, EH111-8, EH26-7, O157-9, O157-19, O157-37, q1701, q1704, q1705, q1716, q1718, q1724, q1726, q1727, q1728, and q1731 exhibited moderate D values; and loci EHC-1, EHC-5, and EHC-6 exhibited high D values. Variations at loci EH111-14, O157-19, q1718, and q1726 were dominantly observed in serogroup O103, though the D values were moderate. Locus q1718 exhibited a very low frequency of null alleles.

O121: The overall discriminatory power of MLVA43 was higher than that of MLVA17. MLVA43 identified more types than did MLVA17. Only three loci, including EHC-1, EHC-2, and EHC-5, exhibited moderate to high D values in MLVA17. In MLVA43, loci q1704, q1705, and q1725 exhibited moderate D values, while locus q1723 exhibited a high D value, which contributed to the high overall discriminatory power of the MLVA43 scheme. Locus q1723 was ubiquitously amplified, but variations at this locus were dominant in serogroup O121.

O145: The overall discriminatory power of MLVA43 was higher than that of MLVA17. MLVA43 identified more types than did MLVA17. Loci EHC-1, EHC-6, O157-9, O157-17, and O157-37 exhibited moderate to high D values in MLVA17. In MLVA43, loci q1704, q1705, q1707, q1708, q1717, and q1727 exhibited moderate D values; while loci q1710 and q1725 exhibited high D values. Loci O157-17, q1707, q1708, and q1710 were specific for serogroup O145 in terms of both variations and frequency of null alleles.

O165: The overall discriminatory power of MLVA43 was similar to that of MLVA17. MLVA43 identified more types than did MLVA17. Loci EHC-5, EHC-6, and O157-37 exhibited high D values in MLVA17. In

MLVA43, loci q1712 and q1714 exhibited moderate D values, and loci q1711, q1715, q1716, and q1725 exhibited high D values. Variations at loci q1711, q1714, and q1715 were dominant in serogroup O165. Loci q1711 and q1714 were ubiquitously amplified, and locus q1715 was unique to serogroup O165.

O91: The overall discriminatory power of MLVA43 was similar to that of MLVA17. MLVA43 identified more types than did MLVA17. Loci EHC-1, EHC-5, EHC-6, and O157-37 exhibited moderate to high D values in MLVA17. In MLVA43, loci q1704, q1727, and q1730 exhibited moderate D values, and loci q1729 and q1731 exhibited high D values. Variations at loci q1729 and q1730 were observed only in serogroup O91. The frequency of null alleles was low at locus q1729 but high at locus q1730 in serogroup O91.

Ubiquitous or non-functional loci: Loci EHC-1, EHC-2, EHC-5, EHC-6, O157-9, O157-37, q1704, q1705, and q1725 exhibited moderate to high D values in multiple serogroups. The D values of loci EH157-12, O157-3, O157-25, and O157-36 were nearly zero. Loci EH157-12 and O157-25 were ubiquitously amplified in most of the serogroups; and the frequency of null alleles at loci O157-3 and O157-36 was almost one in all the serogroups studied.

Analyses of outbreak-related isolates: Isolates related to the two O103 outbreaks were examined using MLVA43. Outbreaks A and B involved 36 and 18 isolates, respectively. For outbreak A, three MLVA profiles, consisting of 33 isolates, two isolates, and one isolate, were obtained; and they differed from each other at locus O157-9. For outbreak B, two MLVA profiles, one consisting of 17 isolates and the other consisting of one isolate, were obtained; and they differed from each at locus O157-37.

DISCUSSION

STEC infection is one of the most serious issues in relation to public health. Molecular subtyping is an essential tool for differentiating isolates and detecting outbreak clusters. MLVA is one of the major tools for subtyping. MLVA is advantageous owing to its rapidity

Table 3. Characteristics of additional VNTR locus used in this study for non-O157 STEC serogroups

Locus name	Sero-group	Strain ¹⁾	Complete/contig No.	Start site	Strand	Predicted size	Repeat length	Repeat sequence	Offset size	Putative feature
q1701	O103	2015C-3163	Complete	3355162	n	160	8	GCGGGTA	128	Intergenic
q1702	O111	11128	Complete	2133183	n	260	6	CGCCAG	248	MFS transporter <i>ydhP</i>
q1704	O121	FWSEC0006	Complete	2807824	c	500	6	AMAGAW	440	Primosomal protein I
q1705	O103	2013C-3264	Complete	1312318	n	446	12	WTCTTTATCTGT	398	Hypothetical protein
q1707	O145	RM13514	Complete	1512269	n	342	12	TCTTTCTTTTCAG/ GATTCTTTTCAG	306	Hypothetical protein
q1708	O145	RM13514	Complete	2420529	c	262	3	GCT	241	<i>fliC</i>
q1710	O145	RM13514	Complete	5470645	c	540	24	MAACARKYCAWTR ACARTAYSGWK	348	Hypothetical protein
q1711	O165	2013C-4830	Complete	3882504	c	389	7	GGCTTAT	361	Integenic
q1712	O165	2013C-4830	Complete	1630919	c	490	9	ATTAACGCA	454	oxidoreductase
q1714	O165	2013C-4830	Complete	3547437	n	328	8	GATGGCGG	304	<i>adhP</i>
q1715	O165	2013C-4830	Complete	4299938	n	185	6	TCCGGC	149	<i>pduC</i>
q1716	O165	2013C-4830	Complete	205181	c	154	6	AATGAA	136	Intergenic
q1717	O26	FWSEC0001	Complete	922853	c	166	5	TTTGT	136	Hypothetical protein
q1718	O26	11368	Complete	5583703	c	447	8	TGCGAAGG	423	Intergenic
q1718	O103	12009 ²⁾	Complete	5290647	c	447	8	TGCGAAGG		
q1720	O26	11368	Complete	5509606	n	259	8	RAATATAT	211	DNA-binding protein
q1721	O111	JNE080262	contig003	61797	n	490	9	TGAACCAAC	454	Intergenic
q1722	O26	11368	Complete	5576065	n	482	4	GCTG	450	Intergenic
q1723	O121	FWSEC0006	Complete	4893727	c	418	6	TCTCCC	364	Intergenic
q1724	O103	12009	Complete	3051096	n	195	6	GCGCTG	159	<i>hyfF</i>
q1725	O103	12009	Complete	2922510	n	274	9	CAGTTCARC	220	DUF1627 domain-containing protein
q1726	O103	NIPH-11060424	contig01055	2392	c	348	7	TAAGYAA	320	Intergenic
q1727	O103	12009	Complete	1020352	c	565	39	ATMYTGCKGCTGY GGYGSAAACYKGYT GTTGYGGCTGYTG	292	<i>ftsK</i>
q1728	O103	NIPH-11060424	contig01100	82222	n	195	13	TCTCATGGCATGT	143	Putative transporter
q1729	O91	2013C-4404	Complete	17133	n	258	6	GAACAG	234	DNA-binding protein
q1730	O91	JNE160956	contig024	46369	c	352	9	TTGCCCGTA	298	No known function
q1731	O91	2013C-4404	Complete	2851198	n	133	8	ACTACGAA	117	Intergenic

¹⁾: Reference strain for sequence information; start site, strand (normal or complementary), predicted sizes, offset size.

²⁾: The sequence is used for designing R1 primer sequence of q1718.

and high resolution; however, the discriminatory power of MLVA varies among serogroups (5,14). Though the most dominant serogroup in STEC infections is O157, the frequency of detection of non-O157 serogroups have increased in recent years. Among non-O157 serogroups, O26, O111, O103, O121, O45, and O145 are the most frequently isolated serogroups in the United States. Attempts to expand the target serogroups for MLVA have been reported previously (14).

In Japan, more than 3000 cases of STEC infections are reported annually (2). Serogroups O26, O111, O103, O121, O145, O165, and O91 have been regarded

important owing to their high frequency of isolation and association with severe cases accompanied by serious conditions, such as HUS. The main aim of this study was to design an MLVA scheme for differentiating serogroups O103, O121, O145, O165, and O91 from the non-O157 STEC isolates obtained from Japan. In our previous study, we designed an MLVA scheme for serogroups O157, O26, and O111 using 17 loci; in the present study, we added 26 new loci to this scheme for identification of the additional serogroups. We used the MLVA43 scheme for evaluating the non-O157 serogroups. The discriminatory power of MLVA43 was

Table 4. Comparison of discriminatory power of subtyping methods for non-O157 STEC serogroups

	Serogroup	N	Method	#type	D	(95 % CI)
2007-2016	All	184	MLVA43	129	0.991	0.987-0.995
			PFGE95	116	0.971	0.956-0.987
	O103	57	MLVA43	39	0.976	0.956-0.995
			PFGE95	46	0.992	0.987-0.998
	O121	63	MLVA43	37	0.954	0.926-0.983
			PFGE95	19	0.769	0.674-0.865
	O145	17	MLVA43	11	0.882	0.750-1.015
			PFGE95	12	0.956	0.913-0.999
	O165	23	MLVA43	20	0.988	0.972-1.004
			PFGE95	15	0.945	0.889-1.000
2007-2017	All	707	MLVA43	19	0.995	0.981-1.008
			PFGE95	20	1.000	na ¹⁾
	O26	272	MLVA17	396	0.993	0.992-0.995
			MLVA43	468	0.997	0.996-0.998
	O111	38	MLVA17	188	0.994	0.991-0.996
			MLVA43	203	0.995	0.993-0.997
	O103	133	MLVA17	34	0.993	0.983-1.003
			MLVA43	34	0.993	0.983-1.003
	O121	123	MLVA17	66	0.962	0.946-0.977
			MLVA43	73	0.967	0.952-0.982
	O145	50	MLVA17	27	0.888	0.856-0.920
			MLVA43	56	0.968	0.956-0.980
	O165	31	MLVA17	16	0.802	0.710-0.895
			MLVA43	27	0.940	0.905-0.974
	O91	60	MLVA17	24	0.983	0.967-0.998
			MLVA43	27	0.991	0.981-1.002
			MLVA17	42	0.978	0.961-0.995
			MLVA43	49	0.992	0.984-0.999

¹⁾: not available.

comparable to that of PFGE (for all serogroups except O121), which is the traditional “golden standard” method of molecular subtyping. In serogroup O121, the discriminatory power of MLVA43 was superior to that of PFGE.

As compared to the MLVA17 scheme, MLVA43 scheme identified more profiles in all serogroups except in O111. In serogroups O26 and O111, the MLVA17 scheme could sufficiently differentiate the isolates, as the D values exceeded 0.99. However, few of the additional loci, including q1725, exhibited high D values, which can contribute to increasing the discriminatory power of MLVA. Thus, MLVA43 can be used when high discriminatory powers are required for outbreak investigation of serogroups O26 and O111.

In the remaining five serogroups, MLVA43 identified more types than did MLVA17, and raised the D values to 0.94–0.99. As compared to MLVA17, MLVA43 dramatically increased the D values for serogroups O121 and O145. Locus q1723 contributed to the increase in the discriminatory power for serogroup O121. This locus corresponds to locus SVL-23 from the previous study, where it was consistently diverse in serogroup O121 (14). Loci O157-17, q1704, q1705, q1707, q1708, q1717, and q1727 were moderately

diverse, and locus q1710 was highly diverse in O145. Locus O157-17 was thought to be specific to serogroup O157, but this locus was amplified in almost all O145 isolates. Other loci as well as O157-17 used in this study were likely to have contributed to increasing the discriminatory power for serogroup O145.

Serogroups O165 and O91 were highly differentiated by both MLVA17 and MLVA43. The discriminatory power of MLVA17 was mostly dependent on loci EHC-6 and O157-37. As these loci are known to be located on plasmid(s), MLVA43 should be chosen for routine application (5,14).

Furthermore, among the widely distributed EH111-11, EH157-12, EHC-1, O157-34, O157-25, O157-19, q1727, q1714, and q1723 loci, loci with moderate or high D values for all tested serogroups, including O157-34 and q1727, may be useful for inter-serogroup differentiation.

The unit length of tandem repeats at loci q1708 and q1722 were 3 and 4, respectively. This may cause problems in inter-laboratory quality assurance owing to the small unit length (18).

The discriminatory powers of few loci, including q1726, were low for intra- and inter-serogroups. Although loci with moderate and high D values for

Table 5. D values of VNTR loci for non-O157 STEC serogroups

Locus	All	O26	O111	O103	O121	O145	O165	O91
EHC-1	0.885	0.805	0.912	0.790	0.760	0.544	0.000	0.903
EHC-2	0.898	0.918	0.853	0.298	0.584	0.079	0.000	0.000
EHC-5	0.486	0.599	0.292	0.606	0.183	0.040	0.738	0.333
EHC-6	0.605	0.411	0.198	0.618	0.095	0.224	0.912	0.739
EH26-7	0.562	0.681	0.000	0.127	0.000	0.000	0.000	0.033
EH111-8	0.285	0.000	0.337	0.180	0.016	0.040	0.000	0.000
EH111-11	0.101	0.000	0.511	0.000	0.000	0.000	0.000	0.000
EH111-14	0.478	0.007	0.053	0.190	0.000	0.040	0.000	0.066
EH157-12	0.006	0.000	0.000	0.000	0.000	0.040	0.065	0.000
O157-3	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
O157-9	0.690	0.769	0.882	0.277	0.000	0.389	0.000	0.000
O157-17	0.125	0.000	0.000	0.000	0.000	0.259	0.000	0.000
O157-19	0.039	0.000	0.000	0.153	0.000	0.040	0.000	0.000
O157-25	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.066
O157-34	0.481	0.000	0.000	0.127	0.000	0.040	0.000	0.000
O157-36	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
O157-37	0.412	0.312	0.910	0.325	0.080	0.154	0.531	0.550
q1701	0.100	0.000	0.000	0.413	0.000	0.040	0.000	0.000
q1702	0.047	0.029	0.336	0.087	0.000	0.000	0.000	0.000
q1704	0.672	0.420	0.104	0.233	0.364	0.150	0.065	0.381
q1705	0.636	0.118	0.053	0.423	0.222	0.190	0.065	0.000
q1707	0.122	0.000	0.000	0.000	0.000	0.187	0.000	0.000
q1708	0.125	0.000	0.000	0.000	0.000	0.224	0.000	0.000
q1710	0.127	0.000	0.000	0.000	0.000	0.633	0.000	0.000
q1711	0.085	0.000	0.000	0.000	0.000	0.000	0.512	0.000
q1712	0.538	0.000	0.000	0.140	0.000	0.078	0.469	0.033
q1714	0.098	0.036	0.000	0.000	0.016	0.000	0.443	0.000
q1715	0.359	0.000	0.000	0.000	0.000	0.040	0.647	0.000
q1716	0.413	0.007	0.000	0.178	0.016	0.040	0.787	0.000
q1717	0.096	0.194	0.000	0.000	0.000	0.246	0.000	0.000
q1718	0.518	0.106	0.000	0.193	0.000	0.040	0.000	0.000
q1720	0.587	0.684	0.000	0.130	0.000	0.000	0.065	0.000
q1721	0.020	0.000	0.329	0.000	0.000	0.000	0.000	0.000
q1722	0.488	0.755	0.053	0.000	0.000	0.000	0.000	0.000
q1723	0.281	0.007	0.000	0.000	0.858	0.000	0.000	0.033
q1724	0.305	0.007	0.053	0.377	0.000	0.079	0.000	0.000
q1725	0.755	0.656	0.717	0.059	0.212	0.766	0.763	0.033
q1726	0.036	0.000	0.000	0.158	0.000	0.079	0.000	0.000
q1727	0.563	0.007	0.000	0.167	0.079	0.154	0.000	0.239
q1728	0.513	0.000	0.000	0.283	0.000	0.079	0.000	0.033
q1729	0.103	0.000	0.000	0.000	0.000	0.040	0.000	0.666
q1730	0.025	0.000	0.000	0.000	0.000	0.000	0.000	0.271
q1731	0.486	0.029	0.000	0.320	0.000	0.078	0.000	0.505

MLVA Scheme for Non-O157 STEC

Table 6. Frequency of null allele (%) and range of alleles

	All	O26	O111	O103	O121	O145	O165	O91	Allelic range
EHC-1	0	0	0	1	0	2	0	0	3-28
EHC-2	11	0	3	0	0	96	100	0	3-35
EHC-5	71	61	84	62	90	98	42	82	2-25
EHC-6	59	76	8	29	95	88	0	17	3-33
EH26-7	63	6	100	93	100	100	100	98	1-6
EH111-8	9	0	0	1	1	0	0	100	1-13
EH111-11	0	0	0	0	0	0	0	0	2-4
EH111-14	39	0	0	11	100	98	100	97	1-2
EH157-12	0	0	0	0	0	0	3	0	1-2
O157-3	100	100	100	100	100	100	100	100	na ¹⁾
O157-9	52	0	0	85	100	78	100	100	5-21
O157-17	93	100	100	100	100	6	100	100	3-5
O157-19	0	0	0	0	0	0	0	0	0-7
O157-25	0	0	0	0	0	0	0	0	2
O157-34	0	0	0	0	0	2	0	0	1-3
O157-36	100	100	100	100	100	100	100	100	na ¹⁾
O157-37	76	83	13	82	96	92	68	23	0-18
q1701	95	100	100	73	100	98	100	100	3-7
q1702	0	0	0	0	0	0	0	0	2-9
q1704	45	16	95	87	2	92	97	75	1-12
q1705	47	94	0	2	9	4	0	100	2-5
q1707	93	100	100	100	100	8	100	100	3-4
q1708	93	100	100	100	100	6	100	100	6-8
q1710	93	100	100	100	100	6	100	100	3-11
q1711	0	0	0	0	0	0	0	0	2-5
q1712	52	100	100	8	0	96	0	2	2-5
q1714	0	1	0	0	1	0	0	0	2-4
q1715	78	100	100	100	0	98	0	100	2-7
q1716	23	0	100	10	1	98	0	100	2-8
q1717	95	90	100	100	100	86	100	100	3-8
q1718	45	1	100	9	100	98	100	100	2-5
q1720	60	0	100	93	100	100	97	100	1-12
q1721	99	100	82	100	100	100	100	100	3-5
q1722	69	21	97	100	100	100	100	100	2-24
q1723	0	0	0	0	0	0	0	0	3-15
q1724	2	0	0	8	0	2	0	0	2-7
q1725	37	15	50	97	1	30	0	98	2-12
q1726	98	100	100	92	100	96	100	100	5-16
q1727	0	0	0	0	0	0	0	0	1-9
q1728	54	100	100	8	0	2	0	98	1-4
q1729	95	100	100	100	100	98	100	38	2-4
q1730	99	100	100	100	100	100	100	85	3-8
q1731	29	1	0	19	100	4	100	35	1-11

¹⁾: not available.

each serogroup can be selected for analyzing specific serogroups at a low cost, other options need to be considered. For example, q1726 was designed using the sequence of strain NIPH-11060424, which originated in Norway with serotype O103:H25. H25 is a minor H-type of O103 in Japan, while H2 is the dominant H-type. It is plausible that q1726 could exhibit high discriminatory power in settings where O103:H25 is more abundant. Thus, loci with low discriminatory powers should be evaluated in different settings to determine their significance.

The MLVA scheme developed in this study allowed discrimination among serogroups O103, O121, O145, O165, and O91 as well as O26 and O111. Very few single-locus variations were detected in two sets of outbreak-related isolates. Furthermore, the number of isolates tested in this study might be less for a comprehensive evaluation; therefore, in future, this scheme should be tested using diverse isolates. Applicability of this scheme in outbreak investigation can also be evaluated using a large panel of isolates. Nevertheless, the scheme would be useful for routine molecular subtyping of STEC. MLVA is rapid and cost-efficient for routine testing (6). The serogroups O157, O26, O111, O103, O121, O145, O165, and O91 cover approximately 90 % of the STEC isolates in Japan. MLVA17 and MLVA43 schemes are easy to perform and involve only two and four multiplex PCR amplifications, respectively.

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Conflict of interest None to declare.

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