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Multilocus Variable-Number Tandem-Repeat Analysis for Molecular Typing of *Shigella sonnei*▽†

Shiu-Yun Liang,[1] Haruo Watanabe,[2] Jun Terajima,[2] Chun-Chin Li,[1] Jui-Cheng Liao,[1] Sheng Kai Tung,[1] and Chien-Shun Chiou[1,3]*

Central Region Laboratory, Center of Research and Diagnostics, Centers for Disease Control, Taichung, Taiwan[1]; National Institute for Infectious Diseases, Tokyo, Japan[2]; and Institute of Medicine, Chung Shan Medical University, Taichung, Taiwan[3]

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A multilocus variable-number tandem-repeat (VNTR) analysis (MLVA) method was developed and evaluated for the subtyping of *Shigella sonnei* isolates. A total of 26 VNTR loci were identified by exploring the repeat sequence loci in the genomic sequences of *S. sonnei* strains Ss046 and 53G and by testing 536 isolates that had previously been characterized by pulsed-field gel electrophoresis (PFGE). The discriminatory power of MLVA (Simpson's index of diversity [*D*], 0.9524; 95% confidence interval [CI], 0.9373 to 0.9564) for the 536 isolates was significantly higher than that of PFGE (*D*, 0.8882; CI, 0.8667 to 0.9097). MLVA typing with the four and eight most variable loci had *D* values of 0.9468 and 0.9481, respectively, results approaching that of 26 loci. The usefulness of MLVA for outbreak investigation was evaluated using 151 isolates from 10 shigellosis outbreaks and 22 PFGE-indistinguishable isolates collected from nine epidemiologically unrelated events in five different countries. The evaluations indicated that MLVA was a powerful typing tool to distinguish isolates for outbreak investigation and that it exhibited a good discrimination of the 22 PFGE-indistinguishable isolates. Single-locus variants did occur during the outbreak; therefore, *S. sonnei* isolates with MLVA profiles differing at no more than a single locus should be considered part of the same outbreak. The present study suggests that MLVA has the potential to replace PFGE as a standard method of typing *S. sonnei* isolates for disease surveillance and outbreak investigation.

The shigellae are the causative agents of shigellosis, a common diarrheal disease in developing countries. Among the four *Shigella* species, *Shigella flexneri* is the most prevalent in developing countries; in contrast, *Shigella sonnei* is predominant in industrialized countries (6, 10). *S. sonnei* infection in industrialized countries is often associated with food-borne transmission and international travel (4, 9, 11, 20, 23).

Genetic characterization of bacterial strains with a variety of genotyping methods is frequently applied for epidemiological investigation. A number of genotyping methods have been developed for *S. sonnei* (3, 15, 16, 21). Among these genotyping methods, pulsed-field gel electrophoresis (PFGE) has become the standard and has been used to build an international molecular subtyping network, PulseNet, for food-borne disease surveillance (22). PFGE has been proven by PulseNet laboratories to be a powerful tool for the routine subtyping of *S. sonnei* isolates for detecting clusters of infections. However, at times, PFGE is not sufficiently discriminatory to distinguish some of the epidemiologically unrelated *S. sonnei* strains, and PFGE data are not appropriate for clonal analysis of *S. sonnei* strains that have evolved over a period of years. In a previous study, we developed an inter-IS*1* spacer typing (IST) method for the typing of *S. sonnei* isolates (3). IST is less discriminatory than PFGE but is more useful for investigating the genetic relationships among *S. sonnei* strains circulating over a longer time span and for discriminating certain strains that are indistinguishable by PFGE. Even though IST obtained more genetic information from PFGE-indistinguishable isolates, it did not discriminate all of the isolates collected from different epidemiological events (3). Therefore, an alternative method with a high discriminatory ability is needed for disease surveillance and outbreak investigation.

Among the next generation of subtyping methods, multilocus variable-number tandem-repeat (VNTR) analysis (MLVA) has been developed for several bacterial pathogens (12–14, 17, 19). Studies have demonstrated that MLVA has a similar discriminatory power to or higher discriminatory power than PFGE (12, 17). MLVA can be used for the routine subtyping of bacterial isolates for disease surveillance and outbreak investigation (8, 12, 19). The present study aims to develop an MLVA method and evaluate its usefulness in typing *S. sonnei* isolates for the purpose of epidemiological investigation.

* Corresponding author. Mailing address: Central Region Laboratory, Center of Research and Diagnostics, Centers for Disease Control, Taichung, Taiwan. Phone: 886-4-24750452. Fax: 886-4-24750474. E-mail: nipmcsc@cdc.gov.tw.

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MATERIALS AND METHODS

Bacterial strains. *S. sonnei* isolates were collected in central and eastern Taiwan between 1996 and 2005. A collection of 536 isolates was used to compare the discriminatory powers of MLVA and PFGE. Within the collection, 151 isolates derived from 10 shigellosis outbreaks were used to evaluate the relative utility of MLVA in discriminating isolates collected from the outbreaks (Table 1). Each disease outbreak was originally reported to the Taiwan Centers for Disease Control (Taiwan CDC) by local public health departments and was

TABLE 1. Characteristics of 10 shigellosis outbreaks and genotypes of 151 *S. sonnei* isolates from the outbreaks

Outbreak	Date of isolation (yr/mo/day)	Location (county)	No. of isolates	PFGE type[a] (no. of isolates)	IST[a] (no. of isolates)	MLVA type (no. of isolates)
1	1996/2/5–1996/3/10	Hwalien	7	J16N09.0002 (5) J16N09.0003 (2)	IST11 (7)	SS26.73 (7)
2	1998/10/19–1998/11/12	Nantou	9	J16N09.0014 (9)	IST21 (9)	SS26.21 (9)
3	1998/10/31	Taoyuan	8	J16N09.0015 (4) J16N09.0016 (1) J16N09.0129 (1) J16N09.0140 (1) J16N09.0141 (1)	IST18 (8)	SS26.18 (6) SS26.74 (2)
4	1998/11/6	Taoyuan	6	J16N09.0014 (5) J16N09.0096 (1)	IST21 (6)	SS26.21 (5) SS26.75 (1)
5	2000/10/20–1998/11/4	Hwalien	49	J16N09.0019 (17) J16N09.0020 (1) J16N09.0023 (9) J16N09.0024 (1) J16N09.0025 (10) J16N09.0026 (6) J16N09.0027 (1) J16N09.0028 (1) J16N09.0029 (1) J16N09.0131 (1) J16N09.0127 (1)	IST1 (48) IST23 (1)	SS26.1 (3) SS26.66 (43) SS26.129 (3)
6	2001/8/2–2001/8/11	Nantou	17	J16N09.0018 (9) J16N09.0036 (8)	IST3 (16) IST26 (1)	SS26.3 (16) SS26.102 (1)
7	2001/8/9–2001/8/24	Nantou	27	J16N09.0019 (18) J16N09.0023 (3) J16N09.0050 (1) J16N09.0125 (1) J16N09.0147 (4)	IST1 (27)	SS26.1 (22) SS26.111 (2) SS26.125 (2) SS26.130 (1)
8	2001/10/4–2001/10/5	Taoyuan	6	J16N09.0072 (4) J16N09.0086 (1) J16N09.0126 (1)	IST1 (6)	SS26.1 (6)
9	2002/4/21–2002/6/5	Nantou	14	J16N09.0019 (14)	IST1 (14)	SS26.1 (7) SS26.125 (6) SS26.142 (1)
10	2003/12/18–2004/1/9	Taitung	8	J16N09.0118 (8)	IST7 (8)	SS26.7 (8)

[a] Data from a previous study (3).

identified by epidemiological investigation conducted by the Taiwan CDC and local public health departments. The *Shigella* isolates obtained from patients and contact information for each outbreak were sent to laboratories of the Taiwan CDC. Outbreaks often continued over weeks or months. The isolates collected from the 10 outbreaks had previously been characterized using PFGE typing and IST methods (5). Four outbreaks, outbreaks 5, 7, 8, and 9, were caused by an IST1 clone. Another set of 22 isolates with an indistinguishable PFGE (J16N09.0015) pattern collected from nine epidemiologically unrelated events was subjected to MLVA characterization. Twenty of these isolates had been characterized previously by IST (3).

Identification of VNTR loci. The genomes of *S. sonnei* strains Ss046 (GenBank accession no. CP000038) and 53G (obtained from The Wellcome Trust Sanger Institute [http://www.sanger.ac.uk {accessed 1 September 2007}]) were explored for potential VNTR loci using VNTRDB computer software developed by Chang et al. (2). The program, which incorporates the algorithm of the Tandem Repeat Sequence Finder (1), explores tandem-repeat sequence loci from one of the two genomic sequences and then locates the position and counts the number of repeat units for each of the loci in the other. The two genomic sequences are used alternately in turn as the "parent" sequence so that a locus with only one repeat unit in a genome but with two or more repeat units in the other genome will not be missed. The computer searches identified 33 potential VNTR candidates, which contained various numbers of repeats in the two strains. In addition, a locus with five repeat sequence units in both genomes was included to evaluate its potential as a VNTR. In total, 34 VNTR candidates were tested on 10 genetically distinct strains. Twenty-six loci, which had various numbers of repeats in the 10 isolates tested, were considered to be VNTR loci and were chosen for genotyping of the *S. sonnei* isolates.

Preparation of crude bacterial DNA. *S. sonnei* isolates, stored at −70°C, were plated onto tryptic soy agar and incubated overnight at 37°C. A loopful (10 μl) of bacterial growth was removed from the plate, suspended in 100 μl of Tris-EDTA buffer (10 mM Tris-Cl, 1 mM EDTA [pH 8.0]) in an Eppendorf tube, and boiled for 10 min. After centrifugation at 3,700 × *g* for 10 min, the supernatant was transferred into a new tube for use.

MLVA. The primer sets for PCR amplification of the 26 VNTR loci are listed in Table S1 in the supplemental material. The primers were designed using a free program available at the Primer3 website (http://frodo.wi.mit.edu [accessed 1 September 2007]). The forward primer of each primer set was labeled at its 5′ end with an ABI-compatible dye, 6-carboxyfluorescein, NED, VIC, or PET, from Applied BioSystems (Foster City, CA). For multiplex PCR amplification, each 10-μl PCR mixture contained 1× PCR buffer, 3 mM $MgCl_2$, 0.05 to 0.4 μM of each primer, 200 μM of each deoxyribonucleotide, 1.0 unit of the recombinant SuperNew *Taq* DNA polymerase (Jier Sheng Company, Taipei, Taiwan), and 1 μl of DNA template prepared as described above. Seven multiplex PCR combinations (multiplex PCR 1 [M1] to M7) were carried out for the MLVA analysis of each *S. sonnei* isolate. The combinations (and concentrations) of the primers were as follows: for M1, SS12 (0.4 μM), SS14 (0.1 μM), SS16 (0.1 μM), and SS21 (0.1 μM); for M2, SS1 (0.05 μM), SS10 (0.1 μM), SS11 (0.1 μM), and SS22 (0.1 μM); for M3, SS3 (0.1 μM), SS6 (0.2 μM), SS9 (0.05 μM), and SS23 (0.1 μM); for M4, SS5 (0.1 μM), SS7 (0.05 μM), SS8 (0.05 μM), and SS20 (0.1 μM); for M5, SS4 (0.1 μM), SS13 (0.05 μM), SS18 (0.1 μM), and SS25 (0.1 μM); for M6, SS2 (0.1 μM), SS15 (0.1 μM), SS17 (0.1 μM), and SS19 (0.2 μM); and for M7, SS24 (0.1 μM) and SS26 (0.1 μM). The PCR was carried out using a GeneAmp PCR System 9600 (Applied BioSystems). M1 to M6 were performed with a denaturing step at 94°C for 5 min, followed by 30 cycles of amplification at 94°C for 45 s, 55°C for 50 s, and 72°C for 60 s. M7 was performed under the same conditions except that the annealing temperature was set at 62°C.

Prior to size analysis, the fluorescent amplicons were diluted in water in a 1:10 (M7) or 1:100 (M1 to M6) ratio, and 1 μl of the solution was then transferred into 10 μl formamide. After denaturation by heating, amplicons were separated by capillary electrophoresis on an ABI Prism 3130 genetic analyzer with a GeneScan 500 LIZ size standard (catalog number 4322682; Applied Biosystems). Data were collected, and the lengths of the amplicons were determined with GeneScan data analysis software, v. 3.7 (Applied Biosystems). All amplicons of different lengths from each locus were subjected to nucleotide sequence determination to verify the repeat sequence and the number of repeat units in the amplicons. The primers (without the dye label) used for nucleotide sequence determination were the same as the primer sets used for PCR amplification. DNA sequencing was performed using the ABI Prism Big Dye Terminator cycle sequencing ready reaction kit and an ABI Prism 3130 genetic analyzer. The number of repeat units for the 26 VNTR loci and the calculated sizes of amplicons for *S. sonnei* strains Ss046 and 53G (see Table S1 in the supplemental material) were taken as the standards to infer the number of repeat units for each allele of a locus in the isolates tested.

Data analysis. The number of repeat units for each locus was saved as "character type" data in BioNumerics software (version 3.5; Applied Maths, Kortrijk, Belgium) and then subjected to cluster analysis using the minimum spanning tree method. To compare the discriminatory powers of PFGE and MLVA with various combinations of VNTR loci, Simpson's index of diversity (D) and 95% confidence intervals (CI) were calculated according to formulas described previously by Grundmann et al. and Hunter (5, 7). The polymorphism of each locus was represented by Nei's diversity index, calculated as $1 - \Sigma(\text{allelic frequency})^2$.

RESULTS

VNTR loci. In total, 26 VNTR loci were identified after testing 10 genetically distant strains. The characteristics of the 26 VNTR loci are listed in Table 2. Most of the loci had short repeat sequences, which ranged from 5 to 9 bp. Eight loci had only one repeat unit in strain Ss046 or strain 53G. Locus SS1 was unusual: it had one repeat unit in strain 53G but 10 units in strain Ss046. The calculated and observed amplicon sizes of the alleles for each locus are listed in Table S2 in the supplemental material. The copy number of the repeat unit for each allele was confirmed by sequencing, and the nucleotide sequences for the alleles are listed in the supplemental material. By testing 536 isolates, a range of 2 to 20 alleles was found for each of the loci (Table 2). Among the 26 loci, 6 (SS1, SS3, SS6, SS9, SS10, and SS11) were identified with six or more alleles, with a Nei's diversity index ranging from 0.57 to 0.83 (Table 2).

Genotyping. The MLVA profiles for the 536 *S. sonnei* isolates are listed in Table S3 in the supplemental material. MLVA analysis of the 536 isolates resulted in 126 MLVA types, with a D value of 0.9524 (CI, 0.9431 to 0.9616). Using the PFGE method, the isolates were discriminated into 98 PFGE types, with a D value of 0.8882 (CI, 0.8667 to 0.9097). The discriminatory power of MLVA was significantly higher than that of PFGE. MLVA typing with the four most variable VNTR loci (SS3, SS6, SS9, and SS10) identified 106 types in the 536 isolates, with a D value of 0.9468 (CI, 0.9373 to 0.9564). MLVA typing with the eight most variable loci (SS1, SS3, SS6, SS9, SS10, SS11, SS13, and SS23) discriminated the isolates into 114 types, with a D value of 0.9481 (CI, 0.9387 to 0.9576). MLVA typing with another subset of eight loci (SS1, SS3, SS6, SS9, SS11, SS13, SS16, and SS23), which were variable in the 22 PFGE-indistinguishable isolates, discriminated the isolates into 109 types, with a D value of 0.9473 (CI, 0.9377 to 0.9569). The discriminatory powers among the four VNTR combinations were not different statistically.

MLVA subtyping of isolates from outbreaks. One hundred fifty-one *S. sonnei* isolates collected from 10 outbreaks were analyzed. The isolates had been previously characterized using PFGE and IST (3). Of the 10 outbreaks, 6 (outbreaks 3, 4, 5, 6, 7, and 9) were identified with two or more MLVA types among the isolates collected (Table 1). Except for outbreak 9, each of the six outbreaks had a predominant MLVA type. Two main MLVA types were found in the isolates from outbreak 9; in contrast, only one PFGE type was identified in the isolates from outbreak 9 (Table 1). MLVA identified fewer types than PFGE from among the 151 isolates. In total, MLVA identified 15 types while PFGE identified 29 types in the 151 isolates.

Cluster analysis of the MLVA profiles using a minimum spanning tree algorithm demonstrated the usefulness of MLVA in discriminating *S. sonnei* strains collected from dif-

TABLE 2. Characteristics of 26 VNTR loci for *S. sonnei* identified in this study

VNTR locus	Length of repeat unit (bp)	No. of repeat units for strain:		No. of alleles[a]	Allelic diversity[b]
		Ss046	53G		
SS1	7	10	1	11	0.57 (0.72)
SS2	9	2	1	2	0.29 (0.39)
SS3	7	14	16	20	0.83 (0.91)
SS4	7	2	3	2	0.29 (0.39)
SS5	7	4	3	2	0.34 (0.44)
SS6	7	14	13	20	0.83 (0.92)
SS7	7	2	3	2	0.33 (0.44)
SS8	60	1	2	2	0.04 (0.12)
SS9	6	8	9	9	0.61 (0.76)
SS10	6	3	7	6	0.58 (0.74)
SS11	6	4	6	6	0.57 (0.73)
SS12	9	2	3	4	0.36 (0.48)
SS13	6	3	2	4	0.38 (0.5)
SS14	9	2	3	2	0.29 (0.39)
SS15	6	2	3	2	0.34 (0.44)
SS16	17	2	3	3	0.31 (0.45)
SS17	6	2	3	2	0.34 (0.44)
SS18	5	2	3	3	0.28 (0.37)
SS19	5	2	3	2	0.34 (0.44)
SS20	40	2	1	2	0.34 (0.44)
SS21	18	1	2	2	0.27 (0.37)
SS22	11	2	1	2	0.34 (0.44)
SS23	16	3	5	5	0.38 (0.48)
SS24	168	2	1	2	0.34 (0.44)
SS25	135	1	2	2	0.19 (0.27)
SS26	101	4	4	4	0.09 (0.15)

[a] The number of alleles found in the 536 *S. sonnei* isolates tested in this study.
[b] The value in parentheses was calculated from the 126 *S. sonnei* strains with different MLVA profiles identified in this study.

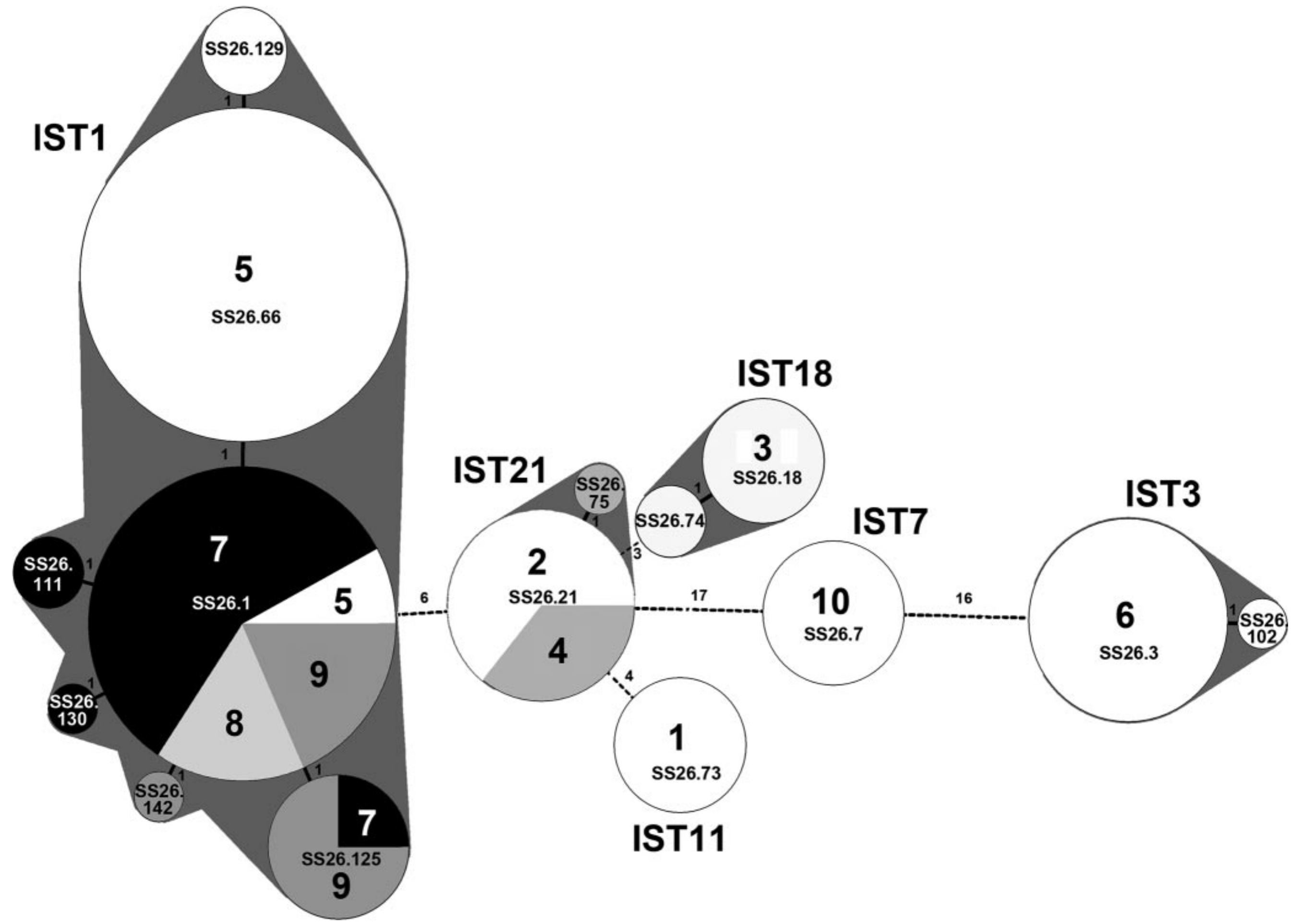

FIG. 1. Minimum spanning tree of the MLVA genotypes for the *S. sonnei* strains collected from 10 shigellosis outbreaks. The clustering was constructed by a minimum spanning tree algorithm. Arabic numerals (1 to 10) and indicate the outbreaks from which the MLVA strains were collected. The circle size is proportional to the number of isolates belonging to the indicated MLVA genotype. MLVA types differing by zero or one VNTR locus are regarded as a group and are marked with gray. Differences in loci between the two MLVA types are numbered. The IST genotypes for the outbreak strains are indicated. MLVA codes are marked in the circles.

ferent outbreaks (Fig. 1). The analysis showed that strains with different IST genotypes were located in distinct clusters. In each of the six outbreaks identified with two or more MLVA types, the minor type(s) differed from the major type by a single locus. Outbreaks 2 and 4 shared a common MLVA type, suggesting that these two outbreaks are epidemiologically related. The two outbreaks occurred in different regions (central and northern Taiwan) but in the same time period.

Four outbreaks (outbreaks 5, 7, 8, and 9) were caused by a common IST1 clone; a strain with the IST1 genotype was detected for the first time in Taiwan in 2000, and the IST1 strain then circulated and caused many outbreaks in central and eastern Taiwan in 2000 to 2003 (24). Isolates from each of the four outbreaks were identified with one to four MLVA types (Fig. 1). SS26.1 was a common MLVA type for the four outbreaks and was the major type for outbreaks 7, 8, and 9. Outbreak 5 was the first outbreak caused by an IST1 strain. SS26.66 was the predominant type for outbreak 5, which was distinguishable from outbreaks 7, 8, and 9.

MLVA of isolates with indistinguishable PFGE patterns. A total of 22 *S. sonnei* isolates were collected from nine epidemiologically unrelated events. The infections occurred in five countries, and the isolates collected were indistinguishable by PFGE with NotI and XbaI. In a previous study (3), 20 of the isolates from eight of the events were characterized and identified with five IST genotypes. The isolates from two events (event 2 [E2] and E3), which were found in Taiwan, and three (E6 to E8) events in Vietnam and Cambodia were not distinguished by IST. In contrast to PFGE, MLVA exhibited a high level of discriminatory power for the isolates. As shown in Fig. 2, the isolates from different events were discriminated into distinct MLVA genotypes. The genetic relationship between the MLVA types constructed using the minimum spanning tree algorithm revealed that the strains from closer geographical locations shared a higher degree of genetic relatedness. For example, the strains from Vietnam and Cambodia shared a higher genetic relatedness than those from other countries. The

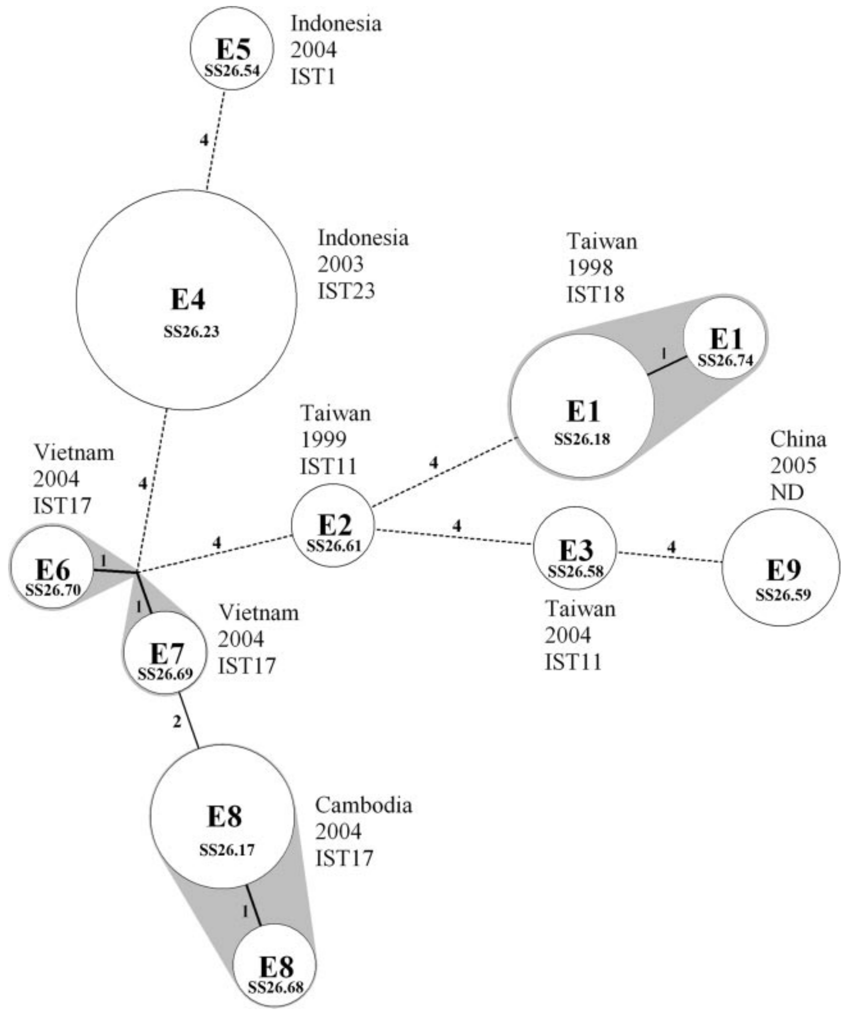

FIG. 2. Minimum spanning tree of the MLVA genotypes for 22 *Shigella sonnei* isolates with indistinguishable PFGE patterns (J16N09.0015) collected from nine epidemiologically unrelated events (E1 to E9) in five different countries. Each event is noted with the country of infection, the year of occurrence, and the IST genotype. The circle size is proportional to the number of isolates belonging to the MLVA genotype. MLVA types differing by zero or one VNTR locus are regarded as a group and are colored gray. The differences in loci between two MLVA types are numbered. The MLVA codes are indicated in the circles.

isolates from E1 and E8 were identified with two MLVA types differing by a single locus.

DISCUSSION

The purpose of developing a novel MLVA method is to find a replacement for PFGE as a routine typing method for disease surveillance and outbreak investigation. PFGE is currently the standard subtyping method for the surveillance of *S. sonnei* infections used in PulseNet laboratories (18). As the result of testing with 536 isolates, MLVA exhibited a significantly higher discriminatory power than PFGE (*D*, 0.9524 versus 0.8882). It has the potential to replace PFGE as the standard typing method in the PulseNet disease surveillance network.

Although a total of 26 VNTR loci were identified, it is not necessary to analyze all the loci for the purposes of disease surveillance and outbreak investigation. The loci that are required in a routine MLVA subtyping scheme will need to be determined by testing more isolates collected from outbreaks in various geographical regions. In fact, the data showed that MLVA typing with the four most variable VNTR loci (SS3, SS6, SS9, and SS10), the eight most variable loci (SS1, SS3, SS6, SS9, SS10, SS11, SS13 and SS23), and the 26 loci had closely comparable discriminatory powers (*D* values of 0.9468, 0.9481, and 0.9524, respectively). The CI values for the three VNTR combinations do not overlap, meaning that the discriminatory power of MLVA typing with the four and eight most variable VNTR loci is not statistically different from that performed with 26 VNTR loci. This study suggests that four to eight loci are sufficient for the subtyping of *S. sonnei* for disease surveillance and outbreak investigation. Since a multiplex PCR can be designed for the amplification of more than four loci, MLVA typing of a *S. sonnei* isolate can be performed with two multiplex PCRs.

MLVA typing of isolates from 10 outbreaks and the strains that were indistinguishable by PFGE also provided insight into the choice of VNTR loci. Six of the 10 outbreaks had two or more MLVA types, and variation occurred in four loci (SS1, SS3, SS6, and SS9). For the 22 PFGE-indistinguishable isolates, variation was detected in eight loci (SS1, SS3, SS6, SS9, SS11, SS13, SS16, and SS23). These eight loci can differentiate the 536 isolates studied into 109 types, with a D value of 0.9473, the result of which approached that using 26 VNTR loci. This set of eight VNTRs and the set of the eight most variable VNTRs share seven loci but differ in the inclusion of SS10 in the former set and SS16 in the latter set.

This study showed that MLVA had good discriminatory power for distinguishing *S. sonnei* isolates from outbreak strains with different IST genotypes. MLVA also provided useful information for distinguishing outbreak 5 from outbreaks 7, 8, and 9. These four outbreaks were caused by a common IST1 clone over a period of 1.5 years. The major MLVA type for outbreak 5 is distinct from the major type for outbreaks 7, 8, and 9. In contrast to PFGE, MLVA identified fewer minor types in the isolates from the 10 outbreaks. In spite of that, PFGE could distinguish only outbreak 8 from outbreaks 5, 7, and 9, which shared a common major PFGE type. It appears that neither MLVA nor PFGE can clearly discriminate the four outbreaks caused by a common IST clone. In a previous study, we showed that IST typing exhibited good discriminatory power for the PFGE-indistinguishable isolates, but it was still not able to discriminate all of the different events (3). In contrast to IST, MLVA can clearly discriminate all of the isolates from the different epidemiological events (Fig. 2), which took place in five countries, including Cambodia, China, Indonesia, Taiwan, and Vietnam, over a period of 7 years (1998 to 2005). This PFGE type might have been circulating in Asia for years. The framework of the strain genome has retained considerable stability. However, the genome did vary over time. These genetic variations could not be detected by PFGE but were detectable with MLVA.

It is contradictory that PFGE is highly discriminatory for *S. sonnei* but at times is not able to discriminate some of the epidemiologically unrelated strains, for instance, the 22 PFGE-indistinguishable isolates characterized in this study. In quite a number of cases, we observed that many new PFGE strains had emerged through sustained transmission of an *S. sonnei* strain. However, it is quite common that a certain PFGE type will continue to be the major one over a period of years. For example, in a previous study, we conducted a PFGE analysis of 291 *S. sonnei* isolates with the IST1 genotype. The study identified 37 PFGE types among the 291 isolates; 51% (148) of the isolates shared a common PFGE genotype (J16N09.0019) (24). When a strain with a stable PFGE genotype is widespread, PFGE will become an ineffective tool for investigation of an outbreak.

Isolates from 6 of the 10 outbreaks were identified as having more than one MLVA type. All of the minor types were single-locus variants of the major type (Fig. 1). Therefore, *S. sonnei* isolates with MLVA profiles differing at no more than a single locus should be considered part of the same outbreak. Single-locus variants occurring during an outbreak are common. They have been observed in other organisms such as *Escherichia coli* O157:H7 (17). In this study, we found two MLVA strains differing by a single locus in a patient from outbreak 9. The two MLVA strains were isolated from the patient at time points separated by 6 days.

In conclusion, an MLVA method with 26 VNTR loci has been developed for typing of *S. sonnei* isolates. It was more discriminatory than PFGE for *S. sonnei*. MLVA can provide useful information for epidemiological investigation of *S. sonnei* outbreaks and of PFGE-indistinguishable *S. sonnei* strains; it has the potential to replace PFGE as a standard typing method for *S. sonnei*. However, study of a greater number of strains from a variety of geographical regions will be needed to determine which loci need to be included in a standard MLVA typing scheme for disease surveillance and outbreak investigation.

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