Original Article

Molecular typing of *Shigella sonnei* isolates circulating in Nanjing, China, 2007–2011

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Abstract
Introduction: Shigellosis is a major public health concern worldwide. This study intended to assess the baseline genotyping data among local *Shigella sonnei* strains spanning over five years.
Methodology: Fifty non-repeat clinical strains of *S. sonnei* isolated from stools of patients in different hospitals in Nanjing, China, were studied. Three subtyping tools, pulsed-field gel electrophoresis (PFGE), multi-locus sequence typing (MLST), and multi-locus variable-number tandem-repeat (VNTR) analysis (MLVA), were used for routinely subtyping local *S. sonnei*.
Results: DNA sequencing only identified two sequence types (STs) among the 50 isolates in the MLST profiles, whereas PFGE and MLVA both showed suitable discriminatory power and yielded 19 and 30 different patterns, respectively. The major PFGE pattern comprised 21 strains isolated from different years. A total of four complexes were identified by MLVA, with the isolates differing by a single locus (single-locus variants).
Conclusions: The *S. sonnei* strains circulating in Nanjing, China, in 2007–2011 originated from different clones with a degree of diversity. Most of the clones were closely related to each other. Overall, the strains were distinguishable by PFGE and MLVA. MLVA based on eight selected VNTR loci represented a more favorable degree of discrimination than did PFGE and may be a reliable complement for PFGE for routine subtyping of *S. sonnei*. The problems of MLST in subtyping regarding *S. sonnei* were also demonstrated.

Key words: *Shigella sonnei*; molecular typing, PFGE; multi-locus sequence typing; multi-locus variable-number tandem-repeat.

Introduction

Shigellosis continues to be a major public health concern worldwide, predominantly affecting children under five years of age in developing countries [1]. Shigellosis is a notifiable disease in China. Although recent surveillance data showed that the overall trend of shigellosis in China is declining, the disease remains more common than in developed countries and presents itself as a serious condition. Shigellosis can be caused by *S. dysenteriae*, *S. flexneri*, *S. boydii*, or *S. sonnei*. The dominant *Shigella* species may be related to the location as well as to the stage of economic development. Among the *Shigella* species of concern, *S. flexneri* accounts for most of the cases in the developing world, and *S. sonnei* is most prevalent in the developed world [1,2]. However, a shift in *Shigella* dominance from *S. flexneri* to *S. sonnei* has been observed in certain newly industrialized areas in Asia, such as Taiwan and South Korea [3-5]. Similarly, increasing rates of *S. sonnei* cases were recorded in economically developed provinces in China between 2001 and 2010 [6].

Although *S. sonnei* is becoming increasingly important as a causative agent of shigellosis in China, there is limited knowledge on the molecular epidemiology of the pathogen. Several molecular typing methods with relatively high discriminatory power, such as pulsed-field gel electrophoresis (PFGE), multi-locus sequence typing (MLST), and multi-locus variable-number tandem-repeat (VNTR) analysis (MLVA), have been used to characterize the strains of *Shigella* species [7-9]. Of these, PFGE is the most widely used and standardized approach for molecular typing of bacterial isolates [10]. MLST was first proposed in 1998 as a definitive and portable typing approach for analyzing bacteria [11]. MLVA analysis of VNTR is a newly devised typing method for characterizing bacterial pathogens, including *S. sonnei* [9]. Molecular characterization of *Shigella* strains originating from defined geographical regions

provides important information to understand its epidemiology and microbiology, and the pathogenesis of *Shigella* infection. This prompted us to further characterize local *S. sonnei* strains at the molecular level for both clinical and epidemiological purposes.

Methodology

Bacterial isolates

A total of 50 *S. sonnei* isolates were collected from hospitals in Nanjing, China, between 2007 and 2011. All the strains were isolated from stool specimens of patients with gastroenteritis. Strain names are specified as location, year, and sample number in sequence. These include five strains from an outbreak in 2011 [12]. Individual isolates were analyzed by standard biochemical tests. Confirmation was made using both API-20E biochemical test strips (bioMérieux SA, Marcy-l'Etoile, France) and a slide agglutination test using *Shigella* group antisera (Denka Seiken Co., Ltd., Tokyo, Japan) according to the manufacturer's instructions.

Preparation of crude bacterial DNA

Crude DNA template was prepared as follows: a pure culture of *S. sonnei* was plated onto Luria-Bertani agar and incubated overnight at 37°C. A loopful of bacterial culture was removed from the plate, suspended in 100 μL of sterile deionized water, boiled for five minutes, and immediately cooled on ice. After centrifugation at 12,000 g for three minutes, the supernatant was transferred into a new tube for use. Crude DNA template was used for MLST and MLVA.

Pulsed-field gel electrophoresis (PFGE)

The standardized PulseNet protocol of the Chinese Center for Disease Control and Prevention was used for PFGE typing (http://www.cdc.gov/pulsenet/pathogens/shigella.html). Briefly, genomic DNA was digested with *Xba*I (Takara Bio, Shiga, Japan), and separated in 1% agarose gels with a contour-clamped homogeneous-field apparatus (CHEF-DRIII system, Bio-Rad, Hercules, USA). *Xba*I-digested *Salmonella* serotype Braenderup H9812 was used as the molecular weight standard. The electrophoretic profiles were visualized by UV illumination, and analyzed using BioNumerics software version 5.1 (Applied Maths, Kortrijk, Belgium). Banding patterns were compared based on the Dice similarity coefficient, and a dendrogram with a setting of 1.5% for optimization and position tolerance of 1.5% for band comparison was constructed by UPGMA (unweighted pair-group method with arithmetic averages) algorithm.

Multi-locus sequence typing (MLST)

Fifteen housekeeping genes were amplified using the primers and polymerase chain reaction (PCR) conditions described below for MLST analysis: *aspC*, *clpX*, *fadD*, *icdA*, *lysP*, *mdh*, *uidA*, *arcA*, *aroE*, *cyaA*, *dnaG*, *grpE*, *mtlD*, *mutS*, and *rpoS*. Loci and primers from the EcMLST database (http://www.shigatox.net/ecmlst) are shown in Table 1. The PCR mixture contained 0.2 μM each of primer, DNA template, 10x PCR buffer 5 μL (Takara Bio, Shiga, Japan), and dNTP 200 μM (Takara Bio, Shiga, Japan) in a volume of 50 μL. Cycling conditions were as follows: initial denaturation at 94°C for 10 minutes, 30 cycles of amplification (92°C for 1 minute, 58°C for 1 minute, 72°C for 30 seconds), followed by 72°C for 5 minutes. The PCR products were sequenced at Shanghai Sangon Biotech. Sequences were aligned and analyzed using DNAStar analysis programs (DNAStar, Madison, USA). The results were submitted to the EcMLST database. Different sequences of a given locus were given allele numbers, and each allelic profile defined a sequence type (ST). New sequence types would be assigned a number for the purpose of the analysis.

Multilocus variable-number tandem-repeat analysis (MLVA)

Eight VNTR loci, SS1, SS3, SS6, SS9, SS10, SS11, SS13, and SS23 reported by Liang *et al.* [9] were used. The genomes of *S. sonnei* strain Ss046 (GenBank Accession No. CP000038) were previously explored for VNTR loci identification [13]. The primers and conditions are indicated in Table 2. Simpson's indices of diversity for MLVA were calculated according to Hunter and Gaston [14]. Three multiplex PCR combinations were carried out: mPCR1 consisted of primers for SS3, SS6, SS9, and SS23; mPCR2 consisted of primers for SS1, SS10, and SS11; mPCR3 consisted of primers for SS13. Each 20 μL PCR mixture contained 10× PCR buffer 2 μL (Takara, Japan), 0.2 mM of each dNTPs, 0.2 μM of each primer, 1 unit of TaqDNA polymerase (Takara, Japan), and 1 μL of DNA template. Cycling conditions were slightly modified: initial denaturation at 94°C for 5 minutes, 30 cycles of amplification (94°C for 45 seconds, 55°C for 50 seconds, 72°C for 60 seconds), followed by 72°C for 10 minutes.

Table 1. MLST primer and size of amplicon

Locus	Primer	Primer sequence(5'-3')	Size of amplicon
aspC	aspC-F4	GTTTCGTGCCGATGAACGTC	594 bp
	aspC-R7	AAACCCTGGTAAGCGAAGTC	
clpX	clpX-F6	CTGGCGGTCGCGGTATACAA	672 bp
	clpX-R1	GACAACCGGCAGACGACCAA	
fadD	fadD-F6	GCTGCCGCTGTATCACATTT	580 bp
	fadD-R3	GCGCAGGAATCCTTCTTCAT	
icdA	icd-F2	CTGCGCCAGGAACTGGATCT	669 bp
	icd-R2	ACCGTGGGTGGCTTCAAACA	
lysP	lysP-F1	CTTACGCCGTGAATTAAAGG	628 bp
	lysP-R8	GGTTCCCTGGAAAGAGAAGC	
mdh	mdh-F3	GTCGATCTGAGCCATATCCCTAC	650 bp
	mdh-R4	TACTGACCGTCGCCTTCAAC	
uidA	uidA-277F	CATTACGGCAAAGTGTGGGTCAAT	658 bp
	uidA-934R	CCATCAGCACGTTATCGAATCCTT	
aroE	aroE-F1	GCGTTGGCTGGTGCTGTTA	362 bp
	aroE-R2	GGGATCGCCGGAATATCACC	
arcA	arcA-F1	GACAGATGGCGCGGAAATGC	552 bp
	arcA-R2	TCCGGCGTAGATTCGAAATG	
cyaA	cyaA-F3	CTCGTCCGTAGGGCAAAGTT	571 bp
	cyaA-R3	AATCTCGCCGTCGTGCAAAC	
dnaG	dnaG-F9	ACCGCCGATCACATACAACT	512 bp
	dnaG-R6	TGCACCAGCAACCCTATAAG	
grpE	grpE-F1	CCCGGAAGAAATTATCATGG	488 bp
	grpE-R4	TCTGCATAATGCCCAGTACG	
mtlD	mtlD-F2	GCAGGTAATATCGGTCGTGG	658 bp
	mtlD-R3	CGAGGTACGCGGTTATAGCAT	
mutS	mutS-F1	GGCCTATACCCTGAACTACA	596 bp
	mutS-R1	GCATAAAGGCAATGGTGTC	
rpoS	rpoS-F3	CGCCGGATGATCGAGAGTAA	618 bp
	rpoS-R1	GAGGCCAATTTCACGACCTA	

Table 2. MLVA primers, number of alleles, and Simpson's index (D)

| Locus	Primer 5'-3'	Dye	Core sequence	Location in SS046	No. of alleles in Ss046	Simpson's diversity index (D)
SS1	TTGCCAGTACACCTCACTCG	HEX	ATGCGCC	1616607-1616676	10	0.82
SS3	CTGGGAGATGAACAGGAGGA	FAM	CATTCAA	3462989-3463086	14	0.88
SS6	GAGTCGCTAAACGCTTGCTT	TAMARA	AGAAAGC	4203622-4203719	3	0.88
SS9	CGCAATCAGCAAAACAAAGA	HEX	TGCAGG	4197029-4197076	8	0.61
SS10	ACGGTGGGCTTTCTCTACCT	FAM	AGAGGA	765727-765710	3	0.43
SS11	CTGGTCCGGGAGATTATCG	TAMARA	CTGACT	266254-266277	4	0.50
SS13	AGACGCTGGCTTATGACGAT	HEX	GCTGGT	2893867-2893884	3	0.30
SS23	CTGGCTTAATGGCTACATAC	ROX	GTTAACGCTTACCTCC	3777764-3777717	3	0.08

The resulting PCR products were sent to Beijing TSingKe Technology for separation by capillary electrophoresis on an ABI 3730XL genetic analyzer with a GeneScan 500 LIZ size standard. Data were collected, and the lengths of the amplicons were determined with GeneMapper version 4.0 (Applied Biosystems, Foster City, USA). Amplicons of different lengths from each locus were subjected to nucleotide sequencing to verify the repeat sequence. The primers used for sequence determination were the same as those used for PCR but without the dye label. Amplicon sizes were converted to copy numbers using BioNumerics software version 5.1 (Applied Maths, Kortrijk, Belgium), and the data was analyzed as described previously [15]. A dendrogram was constructed by UPGMA clustering based on categorical coefficient. Non-approximated confidence interval (CINA) was calculated according to formulas described previously [16].

Results

PFGE analysis of *Xba*I-digested chromosomal DNA of the 50 *S. sonnei* strains yielded 19 reproducible PFGE patterns, ranging in size from approximately 20.5 to 668.9 kb. As shown in Figure 1, one major PFGE pattern, designated type 5, was shared by 21 of the 50 analyzed strains (42%). Type 4 (8%), type 11 (8%), type 7 (6%), and type 9 (6%) were relatively prevalent among the remaining isolates. The above five types comprised 70% of the isolates with similarities of 93.00%, suggesting that most of the strains were closely related to each other despite being isolated in different years. The strains isolated during the same year exhibited similar PFGE patterns. The similarities of the five strains associated with an outbreak in 2011 were more than 97.00%, suggesting a very close relatedness of the epidemiologically related strains. Based on 96% similarity, 10 clusters (A–J) were observed, with the largest containing 60% of the isolates (Figure 1). The similarity index for all groups (A–J) was 83%.

Two novel allele types (*rpoS*, 68; *cyaA*, 34) were found in the MLST profile among the 50 clinical isolates. Moreover, two novel sequence types (ST), ST114 and ST115, which contained the new allele type(s), were identified. The majority of the isolates (49 strains) belonged to the ST complex 114, which contains an allelic profile of 9, 13, 18, 19, 13, 3, 18, 3, 16, 14, 23, 13, 17, 68, and 1, in the order of *arcA*, *aroE*, *aspC*, *clpX*, *cyaA*, *dnaG*, *fadD*, *grpE*, *icdA*, *lysP*, *mdh*, *mtlD*, *mutS*, *rpoS*, and *uidA*. Strain 2008-01 was
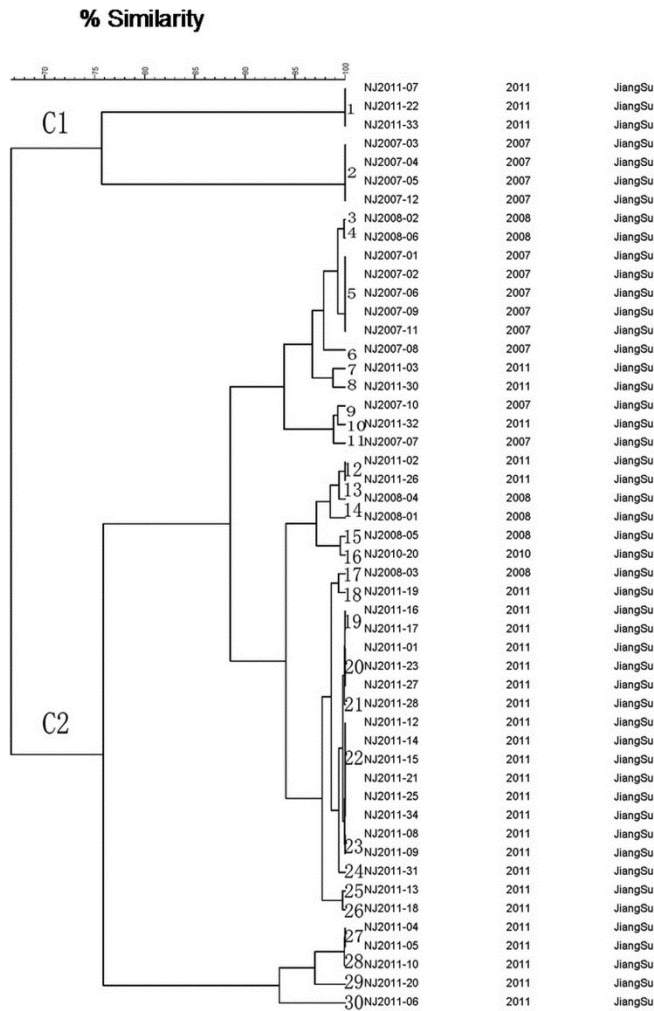
Figure 1. Dendrogram of 50 Nanjing *S. sonnei* isolates based on PFGE patterns generated by the UPGMA clustering method
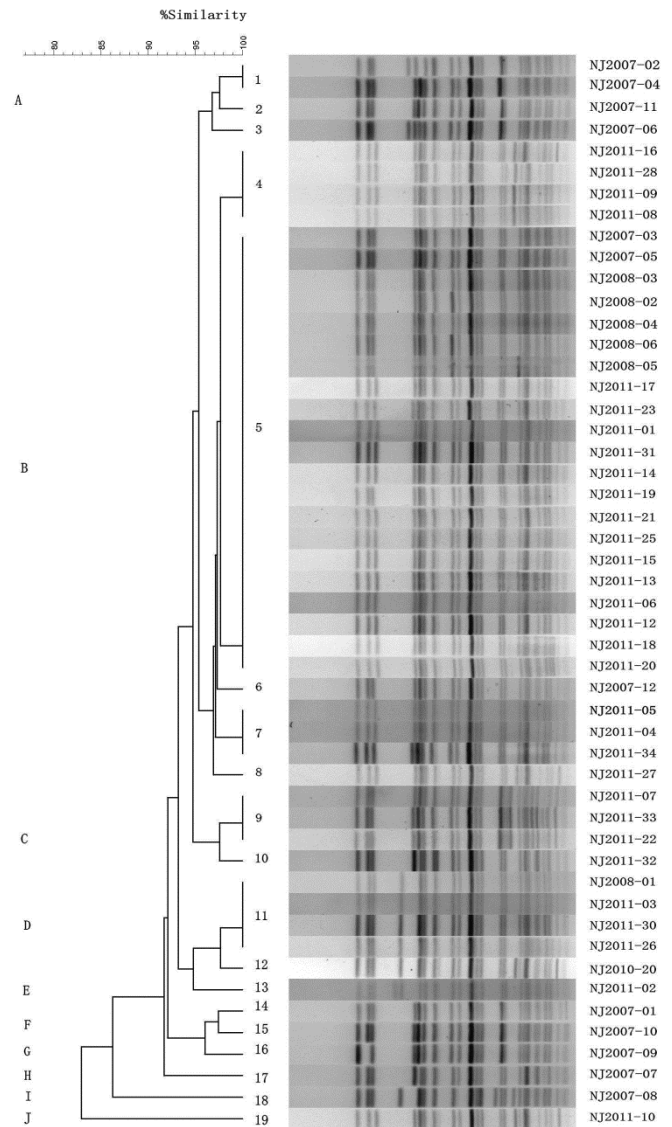

resolved to sequence type ST115, which differed in one allele (*cyaA*) from ST114.

MLVA based on eight VNTR loci was performed to further characterize the isolated *S. sonnei* strains. Overall, the 50 *S. sonnei* strains were discriminated into 30 different MLVA types as shown in Figure 2. Most MLVA types have a distance of two to four loci from each other, mainly in SS1, SS3, SS6, and SS9. A total of four complexes were identified by MLVA where the isolates differed by a single locus (single-locus variants). The five outbreak-related strains were grouped to complex 3 (type 19 and 20, differing at SS3). Single-locus variants occurring during an outbreak were common [9].

Figure 2. Dendrogram generated from 50 Nanjing *S. sonnei* isolates based on MLVA subtyping by the UPGMA clustering method

Figure 3. Dendrogram based on the results of MLVA typing of 50 Nanjing *S. sonnei* isolates, 10 Anhui *S. sonnei* isolates, and 30 Beijing *S. sonnei* isolates
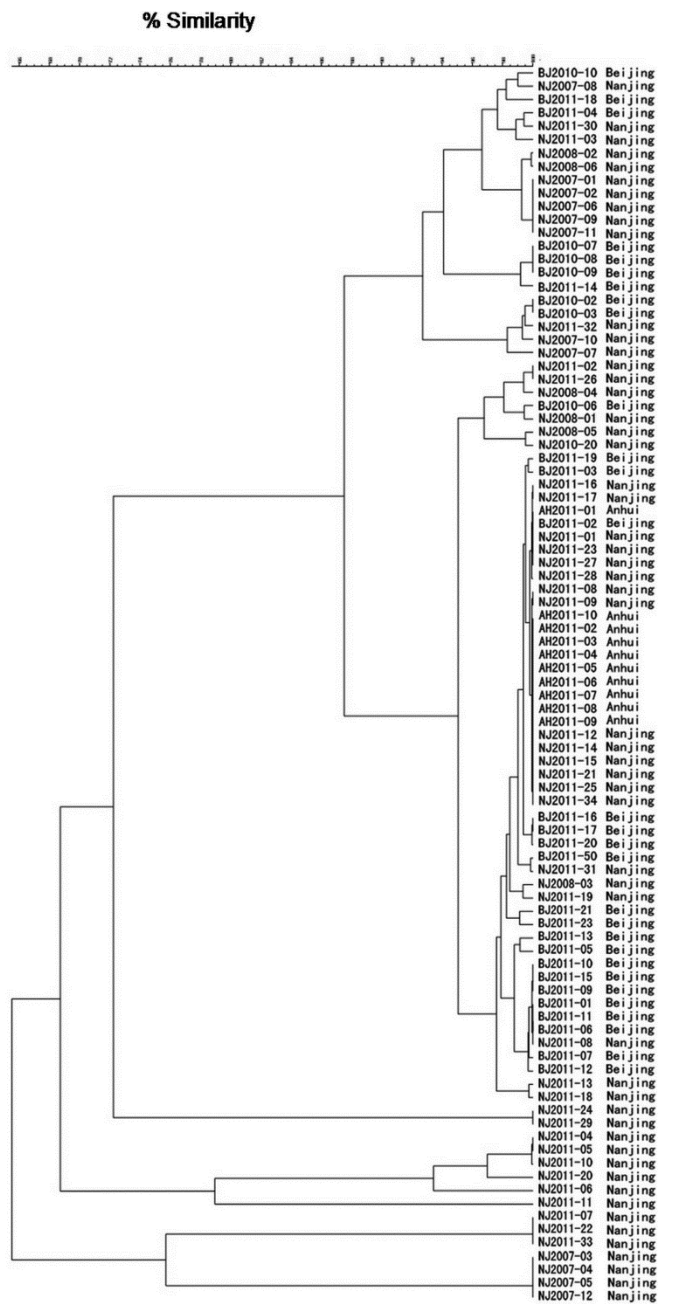
Two main clusters, cluster CI including type 1 and 2, and cluster CII consisting of the remaining types, were observed from the dendrogram generated. *S. sonnei* is one of the major bacterial pathogens causing travel-associated diarrhea [17]. Therefore, the results of this study were compared with MLVA profiles of 40 strains from two other regions in China to determine whether these strains were related. Ten strains obtained from a foodborne outbreak in Anhui province in 2011 and thirty strains isolated from sporadic cases in Beijing between 2010 and 2011 were included. As shown in Figure 2, six isolates from sporadic gastroenteritis cases in Nanjing displayed an identical MLVA pattern, type 22. This particular MLVA pattern was also exhibited by nine strains obtained from patients in a foodborne outbreak in Anhui, a province adjacent to Nanjing (Figure 3). In this outbreak in Anhui, the single strain recovered from leftover food showed a MLVA pattern that was highly related to MLVA 22, differed by one locus at SS6. Nevertheless, the MLVA profiles of the Nanjing isolates differed at two to six VNTR loci from those of Beijing strains. Overall, the combination of the eight VNTR loci with high and low D values was adequate for molecular subtyping of the *S. sonnei* isolates.

Discussion

We confirmed the existence of diverse *S. sonnei* clones and the usefulness of PFGE in local epidemiological studies. We also concluded that most of the isolates were closely related to each other in the period of time studied. Strain 2008-01 assigned to ST 115 by MLST was grouped into cluster D (type 11) in the PFGE profiles. Compared to PFGE profiles, which revealed 19 distinct genetic patterns and 10 clusters, it is apparent that MLST lacks the ability to provide a satisfactory level of discrimination. This low resolution versus PFGE has been recently reported with *Shigella* species [18,8] and with some other species of bacteria as well [19]. Studies suggest that MLST may not be powerful for characterizing closely related strains within a specific serotype due to high sequence conservation of the housekeeping genes [19,20].

MLVA may not be appropriate as a subtyping tool for phylogenetic analysis among different bacterial species or serotypes. Nevertheless, MLVA is useful to establish genetic relatedness among strains of a monomorphic species, such as *S. sonnei* [21]. Though applied in a limited collection of *S. sonnei* isolates, our study suggests that these eight loci were sufficient for the subtyping of *S. sonnei*. Strains isolated five years

apart with highly similar MLVA patterns were observed; for example, strain NJ2007-10 in type 9 and NJ2011-32 in type 10 were closely related to each other (99.0% similarity), revealing the existence of a circulating *S. sonnei* clone with minor genetic changes during the period. Moreover, a number of strains in this study were related to *S. sonnei* from the neighboring Anhui province, and to lesser extent, to those from Beijing. MLVA type 22 was identified in a foodborne outbreak in Anhui province, suggesting that this genotype has been circulating in Nanjing and its neighboring regions since 2011. Moreover, the outbreak strains may be among the most adaptive strains that are prone to spread [22]. MLVA type 19 and 20 are comprised of five outbreak-related strains in Nanjing, and type 22 is comprised of nine outbreak-related strains in Anhui. This indicates more adaptation and greater spreading ability of certain closely related MLVA genotypes.

The results of typing based on MLVA and PFGE were compared. Overall, MLVA identified more types than PFGE among the 50 isolates, and the genetic diversity was higher than that observed in PFGE. Three strains (2011-07, 2011-33, and 2011-22) exhibited identical PFGE patterns (type 9) and were also classified into an identical MLVA pattern (type 1). Nine strains were unique by both PFGE and MLVA. However, those strains that belonged to the same PFGE profile (PFGE type 1, 4, 5, 7, and 11) were distinguishable by MLVA. For example, 21 strains included in one major PFGE pattern (type 5) were further discriminated into 15 minor MLVA genotypes (MLVA type 2, 17, 3, 13, 4, 15, 19, 20, 24, 22, 18, 25, 30, 26, 29), suggesting that MLVA typing exhibited more detailed differences between *S. sonnei* that had similar PFGE patterns. However, the opposite was also found, since isolates with identical MLVA types could correspond to different PFGE types. For example, five isolates belonging to five PFGE patterns (type 1, 2, 3, 14, 16) were clustered into a single MLVA pattern (type 5). Different subtyping methods assessed the genetic characterization in different parts of the chromosome. Variation at the restriction sites may result in differences in PFGE profiles, while gene mutations may affect MLVA repeat locations. A combination of PFGE and MLVA analysis may yield more information about the clonality of *Shigella sonnei*.

Conclusions

In this study, we presented epidemiological trends of *Shigella sonnei* isolates in Nanjing for a period of

time and correlations among three different typing methods. Although PFGE and MLVA both showed suitable discrimination in *Shigella sonnei* subtyping, MLVA exhibited a higher discriminatory ability than did PFGE. Our results were somewhat consistent with the reports that PFGE may not be sufficiently discriminative for clonal research of *S. sonnei* strains that have evolved over very long time periods [9,21]. MLVA would be useful for phylogenetic analysis of PFGE-indistinguishable *S. sonnei* strains. MLST of 15 housekeeping genes showed it to have low discriminatory ability as a subtyping tool for local *S. sonnei* isolates. The observations suggested that the *S. sonnei* circulating in Nanjing belonged to different clones that may be disseminated with minor genetic variations. Most of the clones were closely related to each other. Although our results may not reveal the precise clonal nature of local *S. sonnei* due to the small sample size, the observation has nevertheless described the genetic background of *S. sonnei* circulating in Nanjing in a five-year period. The PFGE banding patterns of these strains have been recorded into the national *S. sonnei* PFGE database, and the information will serve as the starting point for the molecular surveillance of shigellosis in the Nanjing area. MLVA may help to further differentiate subtypes due to its higher discrimination. Maintaining a local *S. sonnei* database may be useful to not only monitor changes in the genetic diversity of isolates over years, but also to detect diffuse outbreaks. Moreover, if patterns of more outbreak strains are supplemented, it is possible to confirm certain subtypes that are prone to spread and are associated with multiple unrelated outbreaks. The identified clones may pose higher risks of causing illness or outbreaks, referred to as a public health risk. Overall, the data will provide a useful typing resource which will therefore help address clinical and epidemiological issues regarding *S. sonnei* in this area.

Acknowledgements

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