Use of multilocus variable-number tandem repeat analysis in molecular subtyping of *Salmonella enterica* serovar Typhi isolates

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We evaluated 11 variable number tandem repeat (VNTR) markers for the epidemiological investigation of *Salmonella enterica* serovar Typhi (*S. Typhi*) infection and compared the results to those obtained by PFGE. PFGE, using one or two restriction enzymes (*Xba*I and *Bln*I), was insufficient to differentiate between some isolates that were epidemiologically unlinked. Multilocus variable-number tandem repeat analysis (MLVA)-8, based on analysis of the eight most variable VNTRs, displayed a high level of discrimination when distinguishing between epidemiologically unlinked isolates that could not be discerned by PFGE with two enzymes. An MLVA-8 typing scheme could be implemented as a routine subtyping tool for the epidemiological investigation of *S. Typhi* infections. Because seven of the 11 VNTRs are highly variable, the VNTR markers may only be useful in determining genetic relationships among very closely related isolates in short-term epidemiological studies and not for discerning *S. Typhi* clones.

Received 16 August 2011
Accepted 7 October 2011

INTRODUCTION

Salmonella enterica subspecies *enterica* serovar Typhi (*S. Typhi*) causes typhoid fever, a disease transmitted primarily through the faecal–oral route. Typhoid fever is most prevalent in South-Central and South-East Asia, causing an estimated 22 million cases and 0.22 million deaths in the year 2000 (Crump *et al.*, 2004). In developed countries, the incidence of typhoid fever has declined markedly and it is now predominantly associated with travellers returning from endemic areas (Connor & Schwartz, 2005).

Characterization by various genotyping methods can provide useful information when trying to assess genetic connections among isolates for short- and long-term epidemiological investigations (Spratt, 2004). Highly discriminatory typing methods for the differentiation of closely related isolates, such as those derived from a very recent common ancestor over a period of months or years, are suitable for the investigation of short-term epidemiology,

such as disease outbreaks and disease surveillance (Hyytiä-Trees *et al.*, 2007). Typing methods, such as multilocus sequence typing (MLST) (Maiden *et al.*, 1998), that can distinguish between clones among isolates evolved over a period of decades are appropriate for long-term epidemiological studies, for example the global spread of hypervirulent clones of rapidly evolving *Neisseria meningitidis* (Caugant *et al.*, 1986; Chiou *et al.*, 2006). MLST and single nucleotide polymorphism analysis have been used for evolutionary and global epidemiology studies of *S. Typhi* (Kidgell *et al.*, 2002; Octavia & Lan, 2007; Roumagnac *et al.*, 2006). PFGE is a highly discriminatory method for the differentiation of most bacterial pathogens. It has been widely used as a subtyping tool for the investigation of disease outbreaks (Hyytiä-Trees *et al.*, 2007) and it was also adopted as a standard subtyping tool for an international foodborne disease surveillance network (Swaminathan *et al.*, 2006). However, in some cases, PFGE is insufficient in distinguishing between epidemiologically unlinked isolates of genetically monomorphic pathogens, such as *Shigella sonnei* (Liang *et al.*, 2007), *Escherichia coli* O157:H7 (Noller *et al.*, 2003), *Salmonella enterica* serovar Enteritidis (Boxrud *et al.*, 2007) and *Salmonella enterica* serovar Typhimurium (Chiou *et al.*, 2010; Torpdahl *et al.*, 2007). On the other

Abbreviations: CI, confidence interval; DI, discriminatory index; MLVA, multilocus variable-number tandem repeat analysis; MST, minimum spanning tree; TR, tandem repeat; VNTR, variable number tandem repeat.

hand, PFGE is too discriminatory to be an appropriate typing tool for the investigation of the global spread of bacterial clones (Spratt, 2004).

Multilocus variable-number tandem repeat analysis (MLVA) is a well-known typing method that has been successfully developed for many bacterial species or for serovars within a species (Liang *et al.*, 2007; Liao *et al.*, 2006; Lindstedt *et al.*, 2003; Marsh *et al.*, 2006; Noller *et al.*, 2003). Based on the analysis of multilocus variable-number tandem repeats (VNTRs), MLVA can be an extremely powerful method for distinguishing between closely related isolates in the investigation of disease outbreaks (Liang *et al.*, 2007; Noller *et al.*, 2003; Torpdahl *et al.*, 2007).

S. Typhi is a genetically monomorphic bacterial pathogen (Kidgell *et al.*, 2002). In our experience, PFGE is not discriminatory enough, in some cases, to distinguish between epidemiologically unlinked *S. Typhi* isolates. In the present study, we screened the released genomic sequences of *Salmonella enterica* serovars Typhi and Paratyphi A for VNTRs. We assessed the diversity of the VNTRs and compared the discriminatory power of MLVA, based on various combinations of VNTRs, against that of PFGE. Finally, we investigated the usefulness of MLVA data in the epidemiological study of *S. Typhi* infection.

METHODS

Bacterial isolates. Bacterial isolates were collected in the Central Region Laboratory, Center for Research and Diagnostics, Centers for Disease Control, Taiwan. In total, 125 *S. Typhi* isolates were collected between 1996 and 2009 (Table 1). Of the 125 *S. Typhi* isolates, 38 were recovered from patients returning from other countries, including Bangladesh (1 isolate), Cambodia (9), China (3), France (1), India (2), Indonesia (18), Myanmar (2), Philippines (1) and Thailand (1). Isolates that had no epidemiological record identifying country of origin were considered as having originated in Taiwan.

PFGE. The PulseNet PFGE protocol for *Salmonella* and other enterobacteria (Ribot *et al.*, 2006) was used for PFGE analysis, except that a smaller amount of *Xba*I (10 U) and *Bln*I (16 U) was used for the digestion of each slice.

Identification of tandem repeat loci with VNTR potential. *S. Typhi* and *S. Paratyphi A* are more closely related at the DNA level than other *Salmonella* serovars, as indicated by whole genome sequence comparisons (McClelland *et al.*, 2004). The genomic sequences of *S. Typhi* strain CT18 (GenBank accession no. AL513382), *S. Typhi* strain Ty2 (accession no. AE014613), and *S. Paratyphi A* strain ATCC9150 (accession no. CP000026) were available at the beginning of this study. To explore as many VNTRs as possible in the development of an MLVA method for *S. Typhi*, the genomic sequence of *S. Paratyphi A* strain ATCC9150 was included in the *in silico* searches using the VNTRDB computer software (Chang *et al.*, 2007). Tandem repeat (TR) sequences that were polymorphic among the three genomes, had >90% sequence similarity and had no indels within the repeat sequence array were selected and evaluated with 11 distantly related *S. Typhi* isolates. The selected TRs that were easily amplified by PCR and had only one amplicon in the isolates tested were further examined for allelic diversity among the 125 *S. Typhi* isolates.

PCR. To perform the PCR, primers for the TRs were designed using the free Primer3 program (<http://frodo.wi.mit.edu/primer3/>). PCRs were carried out using a GeneAmp PCR System 9600 thermocycler (Applied Biosystems). For amplification by PCR, crude bacterial DNA was prepared using a boiling method as described previously (Liang *et al.*, 2007). Each 10 µl PCR mixture contained 1 × PCR buffer, 1% DMSO, 3 mM MgCl₂, 25–100 nM each primer, 200 µM each deoxyribonucleotide, 1 U recombinant SuperNew Taq DNA polymerase (Jier Sheng Company) and 1 µl DNA template prepared by the boiling method. PCR was performed with an initial denaturing step at 94 °C for 5 min, followed by 30 cycles of amplification at 94 °C for 30 s, 55 °C for 60 s and 72 °C for 30 s, and an extension step at 72 °C for 5 min. Occasionally, for some loci, no DNA product was amplified in the multiplex PCR. In these cases, amplification was performed again in a singleplex PCR. For strains where certain TR loci were not amplified when using genomic DNA prepared by the boiling method, genomic DNA was extracted using a commercial kit (GeneAid). The primer sequences for the 11 identified VNTRs are listed in Table 2.

Analysis of PCR amplicons. PCR products were separated by capillary electrophoresis on an ABI Prism 3130XL Genetic Analyzer (Applied Biosystems). The sizes of the amplicons were determined using GeneScan data analysis software version 3.7 (Applied Biosystems) based on the GeneScan 500 LIZ size standard markers. All amplicons with different sizes from each locus were subjected to nucleotide sequence analysis to verify the repeat sequence and the number of repeat units within the amplicons.

Data analysis. PFGE images were analysed using the fingerprint analysis software BioNumerics version 4.5 (Applied Maths). A PFGE genotype was defined as a PFGE pattern with one or more DNA bands different from the others. The number of repeat units for each allele was converted from the amplicon sizes and saved as a 'character type' in the BioNumerics database. An allele that contained a deletion close to a repeat region at Sty20 was assigned to a character type based on the fragment size (bp). The VNTR profiles were subjected to cluster analysis using the minimum spanning tree (MST) and categorical coefficient algorithms provided in the BioNumerics software. The creation of hypothetical types (missing links) was permitted in order to introduce hypothetical types, the group minimum size was set at 2 and the maximum neighbour distance was set at 1. To compare the discriminatory power of PFGE and MLVA based on combinations of various numbers of VNTRs, the discrimination index (DI) (using Simpson's index of diversity) and 95% confidence interval (CI) were calculated using formulas as described previously (Grundmann *et al.*, 2001; Hunter, 1990). The degree of polymorphism for each VNTR was indicated by Nei's diversity index, calculated as $1 - \sum (\text{allelic frequency})^2$.

RESULTS AND DISCUSSION

VNTRs and allelic diversity

To explore as many VNTRs as possible, we used the computer program VNTRDB (Chang *et al.*, 2007) to explore TRs with VNTR potential from the genomic sequences of two *S. Typhi* and one *S. Paratyphi A* strains. Forty-five TRs that had >90% repeat sequence similarity and no indel site within the repeat region were selected for preliminary evaluation. The primers for the 45 TRs were examined using a panel of 11 distantly related *S. Typhi* isolates. The primer screening process selected 34 TRs that were easily amplified by PCR and had only one amplicon in the

Table 1. *Salmonella enterica* serovar Typhi isolates used in this study

Isolate ID	Isolation date	PFGE- <i>Xba</i> I	PFGE- <i>Bln</i> I	MLVA type*	PFGE cluster/ Epi cluster	Country of origin
04-783550	2009/1/25	X.129	A.01	M.125	C1	Indonesia
04-916594	2009/6/11	X.129	A.01	M.034	C1	Indonesia
ST10867	1998/6/11	X.001	A.01	M.089	C2	Indonesia
ST15788	1999/6/28	X.001	A.01	M.076	C2	
ST16676	1999/8/21	X.001	A.01	M.161	C2	Indonesia
N04.0032	2004/1/23	X.001	A.01	M.037	C2	
S05.0884	2005/5/4	X.001	A.01	M.122	C2	Indonesia
C06.1398	2006/7/31	X.001	A.01	M.065	C2	Indonesia
N04.0024	2003/12/27	X.041	A.12	M.124	C3	Indonesia
N04.0030	2004/9/3	X.041	A.12	M.036	C3	Indonesia
04-480280	2009/5/8	X.041	A.12	M.126	C3	Indonesia
04-925983	2009/5/10	X.136	A.71	M.053	C4/Epi-1	
04-356988	2009/5/17	X.136	A.71	M.053	C4/Epi-1	
NA07.321	2007/8/31	X.098	A.48	M.017	C5/Epi-2	
NA07.381	2007/9/14	X.098	A.48	M.017	C5/Epi-2	
C05.0088	2005/1/14	X.016	A.15	M.084	C6	
N05.0022	2005/7/30	X.016	A.15	M.112	C6	
ST18659	2000/2/7	X.016	A.14	M.026	C7	
N04.0594	2004/2/13	X.016	A.14	M.130	C7	
04-139944	2008/9/18	X.106	A.15	M.146	C8	
04-750709	2009/5/2	X.106	A.15	M.147	C8	
ST11628	1998/9/7	X.022	A.19	M.151	C9	
N04.0025	2004/1/3	X.022	A.19	M.157	C9	
NK07.052	2007/8/15	X.022	A.19	M.040	C9	
NB07.038	2007/8/20	X.022	A.19	M.153	C9	
CG07.254	2007/9/29	X.022	A.19	M.155	C9	
04-139907	2008/5/19	X.022	A.19	M.040	C9	
04-043321	2008/5/30	X.022	A.19	M.041	C9	
NK07.068	2007/-/-	X.022	A.19	M.152	C9	
04-289220	2008/-/-	X.022	A.19	M.042	C9	
N05.0014	2005/7/4	X.058	A.13	M.027	C10	
C06.2070	2006/11/6	X.058	A.13	M.027	C10	
S04.3328	2004/8/23	X.058	A.13	M.029	C10/Epi-3	
CA04.003	2004/1/28	X.007	A.04	M.024	C11	
C04.0342	2004/2/5	X.007	A.04	M.023	C11	
N04.0028	2004/10/20	X.007	A.04	M.021	C11	
NA07.528	2007/11/26	X.007	A.04	M.068	C11	
04-764625	2009/5/13	X.007	A.04	M.073	C11	
S04.2684	2004/7/17	X.057	A.11	M.096	C12/Epi-3	
S04.2685	2004/7/17	X.057	A.11	M.097	C12/Epi-3	
S04.2768	2004/7/22	X.057	A.11	M.029	C12/Epi-3	
N05.0009	2005/6/24	X.067	A.03	M.133	C13/Epi-4	
N05.0011	2005/6/29	X.067	A.03	M.038	C13/Epi-4	
N05.0018	2005/7/14	X.067	A.03	M.004	C13/Epi-4	
N05.0021	2005/7/18	X.067	A.03	M.038	C13/Epi-4	
N05.0008	2005/6/19	X.064	A.02	M.004	C14/Epi-4	
N05.0010	2005/6/27	X.064	A.02	M.004	C14/Epi-4	
N05.0015	2005/6/29	X.064	A.02	M.004	C14/Epi-4	
N05.0013	2005/6/30	X.064	A.02	M.005	C14/Epi-4	
N05.0016	2005/7/7	X.064	A.02	M.005	C14/Epi-4	
N05.0019	2005/7/20	X.064	A.02	M.004	C14/Epi-4	
N05.0020	2005/7/21	X.064	A.02	M.005	C14/Epi-4	
N05.0007	2005/6/21	X.065	A.34	M.005	Epi-4	
N05.0012	2005/6/30	X.063	A.10	M.131	Epi-4	
N05.0017	2005/7/15	X.066	A.33	M.005	Epi-4	

Table 1. cont.

Isolate ID	Isolation date	PFGE- <i>Xba</i> I	PFGE- <i>Bln</i> I	MLVA type*	PFGE cluster/ Epi cluster	Country of origin
N04.0040	2004/3/13	X.035	A.66	M.011	C15/Epi-5	Cambodia
N04.0042	2004/4/13	X.035	A.66	M.011	C15/Epi-5	Cambodia
N04.0010	2004/4/15	X.035	A.66	M.011	C15/Epi-5	Cambodia
N04.0007	2004/3/20	X.035	A.64	M.050	Epi-5	Cambodia
NM04.078	2001/5/6	X.037	A.56	M.175	C16	Myanmar
N04.0033	2004/9/16	X.037	A.56	M.216	C16	
ST4256	1996/10/14	X.008	A.08	M.094		
ST7418	1997/6/27	X.014	A.26	M.082		
ST8856	1997/10/30	X.005	A.40	M.139		
ST9093	1997/11/29	X.033	A.62	M.249		India
ST9148	1997/12/13	X.022	A.20	M.154		
ST10070	1998/3/8	X.031	A.32	M.181		
ST10136	1998/3/14	X.010	A.46	M.149		
ST11052	1998/7/1	X.036	A.67	M.083		
ST11786	1998/10/5	X.034	A.61	M.092		Cambodia
ST14115	1998/12/10	X.006	A.41	M.138		Cambodia
ST14969	1998/12/30	X.011	A.24	M.176		Indonesia
ST15395	1999/3/21	X.009	A.06	M.061		France
ST18361	1999/12/6	X.180	A.21	M.165		
ST18600	2000/1/20	X.040	A.42	M.243		Thailand
ST20564	2000/9/11	X.030	A.44	M.252		
ST20567	2000/9/20	X.022	A.23	M.156		Indonesia
ST20608	2000/9/24	X.003	A.24	M.180		Indonesia
ST20882	2000/11/22	X.004	A.31	M.136		Indonesia
ST21149	2001/1/29	X.013	A.45	M.244		China
ST21562	2001/3/28	X.025	A.35	M.072		Indonesia
ST22061	2001/5/21	X.015	A.25	M.081		
ST28481	2002/7/13	X.186	A.76	M.150		
ST30227	2002/11/15	X.020	A.37	M.090		Philippines
NM04.016	2003/7/12	X.042	A.57	M.224		
ST33416	2003/10/23	X.037	A.59	M.183		
ST33681	2003/11/26	X.017	A.43	M.098		
N04.0004	2003/12/31	X.038	A.51	M.135		
S04.0039	2004/1/2	X.061	A.55	M.247		Indonesia
N04.0087	2004/1/7	X.039	A.52	M.058		
N04.0187	2004/1/13	X.021	A.16	M.025		
N04.0265	2004/1/14	X.001	A.38	M.080		Cambodia
S04.0152	2004/1/14	X.062	A.09	M.128		
C04.0728	2004/4/23	X.026	A.28	M.250		
N04.0034	2004/5/6	X.045	A.65	M.234		Bangladesh
N04.0019	2004/5/17	X.052	A.54	M.145		Cambodia
S04.1788	2004/5/19	X.045	A.61	M.047		Cambodia
C04.0905	2004/5/24	X.045	A.64	M.232		
N04.0018	2004/6/27	X.046	A.61	M.233		
S04.2422	2004/7/1	X.043	A.22	M.046		
N04.0031	2004/9/20	X.053	A.01	M.134		
NK04.080	2004/10/10	X.047	A.07	M.069		Cambodia
N04.0027	2004/11/6	X.056	A.50	M.101		
N04.0026	2004/11/9	X.050	A.49	M.095		
S05.1491	2005/7/4	X.055	A.30	M.093		Cambodia
C05.1536	2005/8/2	X.012	A.24	M.043		
C05.1632	2005/8/10	X.068	A.27	M.115		
EA05.036	2005/8/21	X.054	A.47	M.109		Cambodia
CG06.001	2006/1/3	X.071	A.35	M.071		
CA06.017	2006/3/8	X.072	A.18	M.172		

Table 1. cont.

Isolate ID	Isolation date	PFGE- <i>Xba</i> I	PFGE- <i>Bln</i> I	MLVA type*	PFGE cluster/ Epi cluster	Country of origin
C06.0890	2006/4/20	X.073	A.05	M.132		
SG06.091	2006/4/22	X.078	A.15	M.067		
NK06.018	2006/4/25	X.074	A.39	M.246		
C06.1067	2006/5/29	X.076	A.29	M.102		China
SB06.100	2006/10/9	X.037	A.60	M.226		India
C06.1857	2006/10/24	X.080	A.17	M.254		Indonesia
C07.0093	2007/1/22	X.081	A.36	M.110		
ND07.001	2007/6/18	X.085	A.53	M.070		
SB07.062	2007/8/31	X.037	A.73	M.106		
04-417166	2008/5/28	X.103	A.69	M.016		Myanmar
04-347232	2008/5/31	X.078	A.74	M.075		
04-820220	2008/7/30	X.012	A.72	M.179		Indonesia
04-400838	2008/9/17	X.092	A.68	M.174		
04-139904	2008/-/-	X.103	A.70	M.054		
06-091660	2009/6/25	X.098	A.75	M.251		Indonesia

*MLVA type was designated using profiles of 11 VNTRs.

isolates tested. The 34 TRs were further examined for allelic diversity in the 125 *S. Typhi* isolates. Only 12 of the 34 TRs displayed polymorphisms in the 125 *S. Typhi* isolates tested. Ten of the 12 VNTRs had been described previously, including TR1 (Sty41), TR2 (Sty45) and TR5 (Sty42) (Liu *et al.*, 2003); STTR5 (Sty44) (Lindstedt *et al.*, 2003), Sal02 (Sty37), Sal06 (Sty39), Sal20 (Sty40) and Sal10 (Sty43) (Ramisse *et al.*, 2004); and TR4500 (Sty20) and TR4699 (Sty25) (Octavia & Lan, 2009). Two novel VNTRs, Sty2 and Sty3, were discovered in the present study. Sty37 and Sty44 are also variable in *S. Paratyphi* A (Tien *et al.*, 2011). Sty43 (Sal10) had two alleles identified in the 125 isolates; one of the alleles contained the 739 bp insertion sequence element IS200 located immediately upstream of the repeat region. This VNTR was excluded from the data analysis because of its extremely low diversity and the difficulty with which it was analysed by capillary electrophoresis on an ABI Prism 3130XL Genetic Analyzer.

Table 2 lists the characteristics of the 11 VNTRs. The sizes of the repeat units for the 11 VNTRs ranged from 3 to 44 bp. Depending on the locus, the number of alleles varied from 2 to 38 in the 125 isolates characterized, and the alleles contained from 1 up to as many as 43 repeats. Seven VNTRs (Sty20, Sty25, Sty37, Sty40, Sty41, Sty44 and Sty45) were highly diverse, with diversity values of 0.70–0.95. Diversity at a VNTR marker is a function of both the number of alleles and their distribution frequency within a population (Keim *et al.*, 2004). VNTRs that contained higher numbers of alleles tended to have higher diversity values (Table 2) and were more variable (Chiou *et al.*, 2009). Four loci (Sty2, Sty3, Sty39 and Sty42) had low diversity values. The allelic diversity of all VNTRs, with the exception of Sty2, Sty3 and Sty42, were previously evaluated using 73 global *S. Typhi* isolates (Octavia & Lan,

2009) and the diversity levels observed for the VNTRs were similar to those described in this study.

Discriminatory power of MLVA and PFGE

The DIs of various VNTR combinations (corresponding to different multilocus VNTR analyses) and PFGE methods were compared using a panel of 125 isolates. MLVA4, an assay based on analysis of the four most variable VNTRs (Sty25, Sty37, Sty41 and Sty45), displayed a higher DI than PFGE-*Xba*I, PFGE-*Bln*I and a combination of PFGE-*Xba*I and PFGE-*Bln*I for the 125 *S. Typhi* isolates characterized (Table 3). MLVA8, including an additional four VNTR markers (Sty20, Sty40, Sty42 and Sty44), provided further discrimination over MLVA4 and displayed a significantly higher DI than the combination of PFGE-*Xba*I and PFGE-*Bln*I. MLVA11, which includes three VNTRs (Sty2, Sty3 and Sty39) with low-variability in addition to the eight VNTRs of MLVA8, provided no additional discrimination over MLVA8. The combination of MLVA4 with either PFGE-*Xba*I or PFGE-*Bln*I yielded assays with a higher level of discrimination than the three methods alone. However, an assay using a combination of MLVA4 and both PFGE methods displayed the same level of discrimination as that using MLVA4 with just one of the PFGE methods. Similarly, the combination of MLVA8 with either PFGE-*Xba*I or PFGE-*Bln*I displayed a higher level of discrimination than using any of these methods alone and the combination of MLVA8 with either one of the PFGE methods displayed the same level of discrimination as that obtained from MLVA8 or MLVA11 combined with both of the PFGE methods.

Based on the analyses of a small set of highly variable loci, MLVA is sufficient to replace PFGE in resolving closely

Table 2. Characteristics of the 11 VNTRs observed in 125 *Salmonella enterica* serovar Typhi isolates and the primers used for their amplification

VNTR locus (alias)*	Size of repeat unit (bp)	PCR amplicon size range (bp)	No. alleles	Range of repeat units	Allelic diversity	Primers (5'–3')‡
Sty2	6	219–225	2	2–3	0.02	Forward: VIC-TCGGCCAACGATAAGGATAC Reverse: GTGGGCTATCACCGTTGTTC
Sty3	44	197–285	3	1–3	0.09	Forward: PET-TGGTAAATAGCTCCGGAAG Reverse: TTCTCCATGCGCTATACGTT
Sty20 (TR4500)	6	173–240	6	1–11 (173)†	0.70	Forward: NED-CTGGCCCATATTGTGGAGAT Reverse: AAGGTTTCGTAAAGCCGTTGA
Sty25 (TR4699)	6	108–276	25	4–32	0.92	Forward: NED-CGGGCAATTCGAGATAGGTA Reverse: TAGTTTTGGACCTGCCCAT
Sty37 (Sal02)	6	207–357	23	2–27	0.92	Forward: PET-CGATAGACAGCACCAGCAGA Reverse: TCGCCAATACCATGAGTACG
Sty39 (Sal06)	6	174–186	3	5–7	0.15	Forward: 6FAM-TTGGTCGCGGAACATAACTG Reverse: CTTCGTCTGATTGCCACTCC
Sty40 (Sal20)	3	224–266	12	3–17	0.82	Forward: VIC-AATCCGAATCAGCTCACCAG Reverse: GTTTGCGGCTGTTCGTTTAC
Sty41 (TR1 or Sal11)	7	190–274	13	5–17	0.90	Forward: PET-GCCAACGATCGCTACTTTTT Reverse: GCGCATACTACACCGATCAC
Sty42 (TR5 or Sal22)	7	211–218	2	3–4	0.24	Forward: NED-TCGTAGCAGTGAGCCGATAA Reverse: ACTACGCCGAGGATGAACAC
Sty44 (STTR5 or Sal16)	6	178–238	11	5–15	0.84	Forward: VIC-TGCAGTTAATTTCTGCGATCA Reverse: CCTTCCGGATGTATGTGACC
Sty45 (TR2)	8	124–452	38	2–43	0.95	Forward: 6FAM-GAGAGGCTATTATGATTGAACGTG Reverse: TCGTTGAGTCGCCTTTTCTT

*Alias loci, in parentheses, have been previously described: TR1, TR2 and TR5 (Liu *et al.*, 2003); STTR5 (Lindstedt *et al.*, 2003); Sal02, Sal06 and Sal20 (Ramisse *et al.*, 2004); TR4500 and TR4699 (Octavia & Lan, 2009).

†The range of repeat units for allele 173 of Sty20, which has a 7 bp deletion at the 5'-flanking region immediately adjacent to the repeats, is reported instead of that for the fragment size (bp).

‡Multiplex PCRs were performed using primers for the following VNTR loci: M1 (Sty20, Sty37, Sty39 and Sty40); M2 (Sty25, Sty41, Sty44 and Sty45); M3 (Sty2, Sty3 and Sty42).

Table 3. Discriminatory index for the PFGE and MLVA methods based on the analysis of various numbers of VNTR markers

Methods	No. genotypes	DI	95 % CI
PFGE- <i>Xba</i> I	71	0.980	0.971–0.989
PFGE- <i>Bln</i> I	74	0.982	0.973–0.990
PFGE- <i>Xba</i> I + PFGE- <i>Bln</i> I	84	0.986	0.979–0.993
MLVA4	106	0.993	0.986–1.000
MLVA8	109	0.996	0.993–0.999
MLVA11	109	0.996	0.993–0.999
MLVA4 + PFGE- <i>Xba</i> I	110	0.996	0.992–1.000
MLVA4 + PFGE- <i>Bln</i> I	110	0.996	0.992–1.000
MLVA4 + PFGE- <i>Xba</i> I + PFGE- <i>Bln</i> I	110	0.996	0.992–1.000
MLVA8 + PFGE- <i>Xba</i> I	113	0.998	0.996–1.000
MLVA8 + PFGE- <i>Bln</i> I	113	0.998	0.996–1.000
MLVA8 + PFGE- <i>Xba</i> I + PFGE- <i>Bln</i> I	113	0.998	0.996–1.000
MLVA11 + PFGE- <i>Xba</i> I + PFGE- <i>Bln</i> I	113	0.998	0.996–1.000

related isolates of some monomorphic bacteria for the investigation of disease outbreaks (Chiou *et al.*, 2010; Noller *et al.*, 2003; Torpdahl *et al.*, 2007). A previous study (Liang *et al.*, 2007) showed that MLVA, based on only four highly variable VNTR markers, exhibited a level of discrimination that was significantly higher than that provided by PFGE for the *S. sonnei* isolates analysed. The comparisons in this study also indicate that MLVA, based on the analysis of only four highly variable VNTR markers, has a higher DI for distinguishing between *S. Typhi* isolates than PFGE with one enzyme or even PFGE with two enzymes.

Analysis of isolates indistinguishable by PFGE with two enzymes

Of the 125 isolates, 57 in 16 clusters (C1–C16) could not be discriminated by PFGE with two enzymes, *Xba*I and *Bln*I (Table 1). Each of the PFGE clusters contained between two and nine isolates. Ten of the 16 clusters were epidemiologically unlinked. Six clusters (C4, C5, C12, C13, C14 and C15) were epidemiologically related and had been recovered from patients in the same family or from close relatives (Epi-1 and Epi-2), or from patients from the same area within a short time period (Epi-3, Epi-4 and Epi-5) (Table 1). One Epi-3 isolate had a different PFGE type from the other Epi-3 isolates in cluster C12 but shared an indistinguishable PFGE type with the C10 isolates. Three Epi-4 isolates had different PFGE types from other Epi-4 isolates in clusters C13 and C14 and one Epi-5 isolate had a different PFGE type from the other Epi-5 isolates. Epidemiologically unlinked isolates in some of the clusters were recovered over a long period of time. For example, the six isolates in the C2 cluster were recovered during 1998–2006 (Table 1).

The 57 isolates were separated into 40 MLVA4, 43 MLVA8 and 43 MLVA11 types. MLVA8 and MLVA11 displayed a high level of discrimination between the isolates with no epidemiological link. All but two pairs of the epidemiologically unlinked isolates could be distinguished by using

MLVA8 and MLVA11. Two epidemiologically unlinked isolates (NK07.052 and 04-139907) in cluster C9 shared the same MLVA profile (M.040) and two isolates (N05.0014 and C06.2070) in cluster C10 shared a common MLVA type (M.027) (Table 1). Isolates in each of the epidemiologically related clusters C4, C5 and C15 (belonging to Epi-1, Epi-2 and Epi-5, respectively) shared a common MLVA type. Three MLVA types were identified in the three C12 isolates, three MLVA types were identified in the four C13 isolates and two MLVA types were identified in the seven C14 isolates.

In the 16 clusters, variation occurred in eight VNTRs at the following frequencies: Sty45 (in 9 clusters), Sty25 (8), Sty37 (8), Sty41 (6), Sty44 (6), Sty20 (5), Sty40 (5) and Sty42 (1). The variation frequency correlated with the number of alleles ($r^2=0.9259$) and the allelic diversity ($r^2=0.9674$) for the 11 VNTRs in the panel of 125 *S. Typhi* isolates. Accordingly, VNTRs which are more diverse in *S. Typhi* populations are more variable.

The data showed that the PFGE-*Xba*I and PFGE-*Bln*I methods as well as a combination of these methods were insufficient in distinguishing between many epidemiologically unlinked isolates, even though their isolation dates were 8 years apart. Isolates in 10 of the 16 clusters were epidemiologically unlinked but could not be distinguished from one another by the two PFGE methods. In contrast, all but two pairs of epidemiologically unlinked isolates from the 10 clusters were discriminated by MLVA8 and MLVA11.

Genetic relationships and epidemiology

The genetic relationships among the 125 isolates were determined based on the MLVA11 profiles by using the MST algorithm. An identical phylogeny was obtained using MLVA8 profiles because this method displayed the same level of discrimination as MLVA11. As shown in the MST tree (Fig. 1), isolates from each of the five epidemiological clusters (Epi-1–Epi-5) shared either a common MLVA

Fig. 1. Genetic relationships among 125 isolates based on MLVA11 profiles using the MST tree algorithm. The area of the circle is proportional to the number of isolates belonging to the MLVA type. MLVA types for the isolates in the 16 PFGE clusters (C1–C16) are indicated inside the circles. A distance of one locus between two MLVA types is indicated by a thick red line, a distance of two loci by a thin blue line, a distance of three loci by a broken red line and a distance of four loci by a broken blue line. Distances of five loci or greater are marked by a broken grey line. MLVA types differing at one locus are marked with a grey shadow. The MLVA types for isolates in the five epidemiologically related clusters (Epi-1–Epi-5) are marked in different colours. The years of isolation are indicated for two pairs of isolates that emerged 7 and 10 years apart.

profile or close MLVA profiles. The isolates from clusters Epi-1 and Epi-2 shared a common MLVA profile. Multiple MLVA profiles were found in the isolates in clusters Epi-3, Epi-4 and Epi-5 but they were closely linked. The four isolates from cluster Epi-3 possessed three MLVA profiles that differed at *Sty25* with 18, 19 and 20 repeat units. Epi-4 contained 14 isolates that emerged in Northern Taiwan from June 19 to July 21, 2005. These isolates could be divided into five types using PFGE with the two enzymes and five MLVA types that differed at *Sty25* (with 14 and 15 repeat units), *Sty42* (with 3 and 4 repeat units), *Sty44* (with 6 and 7 repeat units) and *Sty45* (with 18 and 19 repeat units). The four Epi-5 isolates differed at *Sty45* with 37 or 38 repeat units.

Typhoid fever is an uncommon disease in Taiwan. In total, 703 cases were confirmed in 1996–2009 with a mean annual incidence rate of 0.22 cases per 10 000 people in the population. At least 35 % of the cases were imported. It was quite unusual that a cluster of 14 cases involving Epi-4 isolates emerged within a county in a 1-month period. These cases should have had a common source of infection

but this was in doubt because multiple PFGE and MLVA types were identified in the isolates. Recently, we detected two MLVA types with a difference of three VNTRs in isolates obtained from a long-term carrier, indicating that the bacteria had evolved into multiple genotypes in the years of carriage. This suggests that Epi-4 isolates with multiple genotypes could come from a common source, a long-term carrier.

Among the epidemiologically unlinked PFGE clusters, two isolates in C10 (recovered on July 4, 2005 and November 6, 2006) as well as two isolates in C9 (recovered on August 15, 2007 and May 19, 2008) shared an identical MLVA type. MLVA types for members of clusters C1, C8 and C9 were closely linked; the MLVA types for members of C1 and C8 had one-locus difference and those for C9 isolates differed by up to three loci between two closest types. For two pairs of isolates that emerged 7 and 10 years apart, their MLVA profiles differed only at one locus (Fig. 1). In contrast, members of clusters C2, C3, C6, C7, C11 and C16 had significantly diversified. The isolates in each of the clusters differed in 4–7 of their VNTRs. The MLVA types for

isolates in clusters C2 and C16 were the most diverse, differing at seven loci.

Isolates that shared indistinguishable PFGE patterns should have closer genetic relationships than those with different PFGE patterns; however, there were distantly linked isolates in PFGE clusters C2, C3, C6, C7, C11 and C16 as shown in the MST based on MLVA11 profiles (Fig. 1). VNTR markers have a wide range of evolutionary rates; therefore, they can be used to investigate genetic relationships among isolates that have evolved over different timescales (Chiou *et al.*, 2009; Pourcel *et al.*, 2004; Wang *et al.*, 2009). Rapidly evolving VNTRs are suitable for investigating genetic relationships among closely related isolates in short-term epidemiological surveillance studies, whereas slowly evolving VNTRs are more useful for establishing clearer clonal relationships among isolates in the investigation of their long-term epidemiology, such as the global transmission of important clones (Chiou *et al.*, 2009). Because seven of the 11 VNTRs for *S. Typhi* were highly variable, the panel of 11 VNTRs is only useful in establishing accurate genetic relationships among very closely related *S. Typhi* isolates, such as Epi-1–Epi-5 isolates.

ACKNOWLEDGEMENTS

This work was supported by the Centers for Disease Control, Department of Health of Taiwan (grant no. DOH98-DC-2022).

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