Jpn. J. Infect. Dis., 66, 180-188, 2013

Original Article

Molecular Characterization of *Salmonella enterica* Serovar Typhimurium Isolated from Human, Food, and Animal Sources in Malaysia

Soo Tein Ngoi[1,2], Bjørn-Arne Lindstedt[3], Haruo Watanabe[4], and Kwai Lin Thong[1,2]*

[1]*Institute of Biological Sciences, Faculty of Science, and*
[2]*Laboratory of Biomedical Science and Molecular Microbiology, Institute of Graduate Studies, University of Malaya, Kuala Lumpur, Malaysia;*
[3]*Division of Infectious Diseases Control, Norwegian Institute of Public Health, Oslo, Norway; and*
[4]*National Institute of Infectious Diseases, Tokyo 162-8640, Japan*

(Received November 6, 2012. Accepted February 7, 2013)

SUMMARY: *Salmonella* Typhimurium is an important nontyphoidal *Salmonella* serovar associated with foodborne diseases in many parts of the world. This organism is the major causative agent of nontyphoidal salmonellosis in Malaysia. We aimed to investigate the genetic profiles of the strains isolated from clinical, zoonotic, and dietary sources in Malaysia using multilocus variable number tandem repeat analysis (MLVA) and pulsed-field gel electrophoresis (PFGE). By focusing on the 5 common variable number tandem repeat (VNTR) loci, we found that PFGE (D = 0.99) was more discriminative than MLVA (D = 0.76). The low MLVA score might be because of a lack of VNTR loci STTR6 (81.0%) and STTR10pl (76.2%). Both subtyping methods suggested that our *S.* Typhimurium strains were largely endemic with limited genetic variation. Furthermore, we observed that biphasic *S.* Typhimurium strains were dominant (99%) and multidrug resistance was prevalent (50%) within our sample pool. The most frequently observed phenotypes were resistance to compound sulfonamides (49%), tetracycline (51%), and streptomycin (52%). In this study, we documented the genetic relationship, antimicrobial resistance characteristics, and flagellar-phase dominance among *S.* Typhimurium strains found in Malaysia.

INTRODUCTION

Salmonella enterica serovar Typhimurium is one of the important nontyphoidal *Salmonella* (NTS) serovars associated with foodborne diseases. Notably, the continuous rise in the number of outbreaks of foodborne illnesses is associated with consumption of *Salmonella*-contaminated raw vegetables and fruits and poorly cooked meats (1,2). In Malaysia, *S.* Typhimurium is the most common causative agent of nontyphoidal salmonellosis (3) and is frequently found in infected patients, contaminated food, and animal sources (2,4). *S.* Typhimurium is the dominant NTS serovar (12.7%) isolated from poultry and livestock in this region (4). The prevalence of *S.* Typhimurium poses a threat to public health. In developing countries, the spread of the pathogenic *S.* Typhimurium is mainly attributed to unhygienic practices during food preparation. Evidence supporting this notion can be found in a study in which *S.* Typhimurium was isolated from ready-to-eat (RTE) food (2).

The emergence of multidrug-resistant (MDR) phenotypes of *S.* Typhimurium has been a major public health concern since the 1990s. Detection of MDR strains in animals were previously reported (5,6); presence of MDR strains in animals can often result in human infection via the consumption of contaminated processed meats (7). The prevalence of these MDR strains raised clinical issues because these strains could complicate the currently available therapeutic options.

S. Typhimurium (antigenic profile 4,[5],12:i:1,2) shows motility by means of peritrichous flagella. These flagella are made up of either of the 2 types of flagellar antigens (H:i and H:1,2), which are encoded by flagellin genes *fliC* and *fljB*, respectively (8). Phase transition of biphasic *S.* Typhimurium is achieved by switching between the expressions of the above-mentioned flagellin genes (9). However, since the mid-1990s, a worldwide increase has been observed in the prevalence of *Salmonella* 4,[5],12:i:–, a monophasic variant of *S.* Typhimurium (8,10). Similar to their biphasic counterparts, many of the monophasic variants also show multidrug resistance (10), and therefore, pose an additional threat to public health. Unfortunately, *Salmonella* 4,[5],12:i:– is antigenically similar to *S.* Typhimurium and can thus be easily misclassified as *S.* Typhimurium during conventional serotyping. In this regard, PCR serotyping is gaining increased popularity in recent years because it affords better precision than that afforded by traditional serotyping (11,12).

Detailed strain identification or strain typing is essential for successful epidemiological investigation of *S.* Typhimurium outbreaks. Currently, pulsed-field gel electrophoresis (PFGE) is considered the gold standard

*Corresponding author: Mailing address: Microbiology Division, Institute of Biological Science, Faculty of Science, University of Malaya, 50603, Kuala Lumpur, Malaysia. Tel: +603-79674437, Fax: +603-79675908, E-mail: thongkl@um.edu.my

technique for molecular subtyping of *Salmonella* and has been widely used in Malaysian studies (2,3). However, the PFGE method has been less successful in discriminating between genetically homogeneous strains (13). The introduction of the multilocus variable number tandem repeat analysis (MLVA) method has addressed this problem (14,15).

In 2003, Lindstedt et al. first proposed the use of 8 variable number tandem repeat (VNTR) loci (STTR1 to STTR8) as the molecular markers, and subsequently, 2 new loci (STTR9 and STTR10pl) were used together with 3 previously reported loci (STTR3, STTR5, and STTR6) to develop a universal MLVA typing scheme (14,15). In another report, Witonski et al. proposed 10 VNTR loci that could simultaneously characterize and discriminate between the *S.* Typhimurium and *S.* Newport strains (16); 3 of these loci were used in the Lindstedt study. PulseNet USA has also developed a 7-locus MLVA protocol for *S.* Typhimurium typing (17), 5 of which overlapped with the Lindstedt study (15). The 5-locus MLVA scheme developed by Lindstedt et al. (15) is the most widely adopted protocol in both European and Australian laboratories (17,18).

Although studies have been conducted on *Salmonella* in other Southeast Asian countries (19–21), currently there is a lack of comprehensive information on the genetic background of *S.* Typhimurium in Malaysia. This study aims to investigate the molecular characteristics of *S.* Typhimurium strains from clinical, zoonotic, and food sources collected from multiple locations in Malaysia. The genetic relationship among the strains was determined by MLVA and PFGE. In addition, the antimicrobial resistance patterns and flagellar phases of *S.* Typhimurium strains were also determined. The data generated provide better understanding of the characteristics of the *S.* Typhimurium population and their transmission dynamics in the region, emphasizing the need for implementation of efficient control measures for disease prevention.

MATERIALS AND METHODS

Bacterial strains: All *S.* Typhimurium strains isolated from 1969 to 2009 were retrieved from glycerol stocks. The strains were obtained from the Laboratory of Biomedical Science and Molecular Microbiology, Institute of Graduate Studies, University of Malaya. However, only 84 viable *S.* Typhimurium strains from clinical ($n = 48$), zoonotic ($n = 16$), and food ($n = 20$) sources were recovered. These strains were isolated from 11 states in Malaysia, namely, Perlis, Kedah, Penang, Perak, Kelantan, Selangor, Pahang, Melaka, Negeri Sembilan, Johor, and Sabah, and the capital city, Kuala Lumpur. Eighty strains were isolated between 2002 and 2009. Four older strains isolated in 1970 ($n = 3$) and 1998 ($n = 1$) were included. All strains were serotyped as *S.* Typhimurium according to the Kauffman-White scheme by the *Salmonella* Reference Laboratory at the Institute for Medical Research, and Veterinary Research Institute in Malaysia. PCR targeting a serotype Typhimurium-specific region was used to confirm the genus and serotype (22).

MLVA analysis: Crude DNA for each strain was prepared by suspending a loopful of bacterial colonies in 50 μl of deionized distilled water and boiling at 99°C for 5 min. After quick centrifugation, 5 μl of the supernatant ($\sim$150 ng of DNA template) was used for PCR. Fluorescence-labeled primers (STTR3-F-**NED**, STTR5-F-**FAM**, STTR6-F-**PET**, STTR9-F-**PET**, and STTR10pl-F-**VIC**) were adapted from the study by Lindstedt et al. (15), with minor modifications on the dye labels so as to not affect the interpretation of results. This set of primers has been widely applied and was sufficiently discriminative for typing *S.* Typhimurium in several European countries (17).

Two multiplex PCR reactions were performed: multiplex-1 (M1), consisting of primer pairs for STTR3 and STTR6, and multiplex-2 (M2), consisting of primers for STTR5, STTR9, and STTR10pl. The M1 PCR master mix consisted of 1 × PCR buffer, 2 mmol·l^{-1} $MgCl_2$, 200 μmol·l^{-1} dNTP mix, 0.3 μmol·l^{-1} of each primer pair, and 2 U of *Taq* DNA polymerase (Promega, Madison, Wis., USA), while the M2 PCR master mix consisted of 1 × PCR buffer, 3 mmol·l^{-1} $MgCl_2$, 200 μmol·l^{-1} dNTP mix, 0.3 μmol·l^{-1} of each primer pair, and 1.5 U of *Taq* DNA polymerase. The PCR thermocycling conditions were performed as described by Lindstedt et al. (15). The PCR products were diluted in a ratio of 1:10, and 1 μl of the diluent was mixed with 10 μl of Hi-Di™ formamide (Applied Biosystems, Foster City, Calif., USA) and 0.3 μl of GeneScan™ 600 LIZ® as an internal size standard (Applied Biosystems). The samples were then denatured for 5 min at 95°C and cooled to room temperature before being subjected to capillary electrophoresis using the ABI Prism® 3130 xl Genetic Analyzer (Applied Biosystems). An electropherogram was generated for each sample; the presence of a VNTR locus appeared as a colored peak. Fragment size was determined by comparing with the size standard. The peak table generated by GeneMapper v4.0 software (Applied Biosystems) was imported into BioNumerics v6.0 software (Applied-Maths, Kortrijk, Belgium). A minimum spanning tree (MST) was constructed using the categorical coefficient and the unweighted pair group method with arithmetic mean (UPGMA) algorithm. The categorical coefficient gives an equivalent weight to a multistate character at any locus regardless of the number of repeats (23).

PFGE analysis: PFGE analysis was performed according to a previously described protocol (24). Genomic DNA was digested with *Xba*I restriction enzyme (12 U per plug) (Promega). *Xba*I-digested *Salmonella* Braenderup (H9812) was used as the DNA size marker. Pulsotypes were analyzed using BioNumerics v6.0 software. The variability of the strains was determined on the basis of the Dice coefficient of similarity (F) and UPGMA clustering analysis at 1.5% position tolerance.

Statistical analysis: The discriminatory power of PFGE and MLVA was determined by Simpson's index of diversity (D) (25). The genetic (allelic) diversity of each VNTR locus was determined by Nei's diversity index using the formula $1 - \Sigma$ (allele frequency)^2. The "null" allele was included in the calculation of allelic diversity.

DNA hybridization: The absence of a VNTR locus was confirmed by DNA hybridization. Approximately 5 μg of genomic DNA was loaded onto a nylon membrane using a PR600 24-slot blot filtration manifold (Hoefer®

Inc., Holliston, Mass., USA) attached to a vacuum pump R-300 (Boeco, Hamburg, Germany). The digoxigenin (DIG) nonradioactive system (Roche Applied Science, Madison, Wis., USA) for DNA hybridization was used. The purified PCR products of the VNTR loci were used as DNA templates to synthesize probes with the PCR DIG Probe Synthesis Kit (Roche Applied Science). The probes targeted the VNTR loci and their flanking regions. The hybridization temperatures for each locus were optimized (54°C for STTR6 and STTR9 and 40°C for STTR10pl). Subsequently, the probe-target hybrids were visualized by a chemiluminescence assay using a chemiluminescent alkaline phosphatase substrate. The nylon membrane was then exposed to an X-ray film, which was subsequently developed and fixed.

Antimicrobial susceptibility test: The antimicrobial susceptibility of the strains was tested using the Kirby-Bauer disk-diffusion method (26), and the zones of inhibition obtained were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (27). The strains were screened for resistance to ampicillin (AMP, 10 μg), amoxicillin-clavulanic acid (AMC, 30 μg), cephalothin (CEF, 30 μg), cefotaxime (CTX, 30 μg), ceftriaxone (CRO, 30 μg), ceftazidime (CAZ, 30 μg), gentamicin (GEN, 10 μg), kanamycin (KAN, 30 μg), streptomycin (STR, 10 μg), tetracycline (TET, 30 μg), ciprofloxacin (CIP, 5 μg), nalidixic acid (NAL, 30 μg), trimethoprim-sulfamethoxazole (SXT, 25 μg), compound sulfonamide (SUL, 300 μg), trimethoprim (TMP, 5 μg), and chloramphenicol (CHL, 30 μg).

PCR serotyping: The primers used were adapted from the study by Cardona-Castro et al. in 2009 (12). The *fliC* primer pairs targeted the phase 1 H:i flagellin gene, while the *fljB* primer pairs targeted the phase 2 H:1,2 flagellin gene. PCR was performed according to a previously described protocol (28). The PCR products were then subjected to gel electrophoresis on a 1.5% agarose gel (Promega) and visualized using the Gel Doc™ XR imaging system (Bio-Rad, Berkeley, Calif., USA) after being stained by ethidium bromide solution (Sigma Aldrich, St. Louis, Mo., USA).

RESULTS

MLVA analyses of *S.* Typhimurium strains: A total of 84 *S.* Typhimurium strains were examined by MLVA using 5 VNTR loci (STTR9, STTR5, STTR6, STTR10pl, and STTR3). Among the 5 commonly tested VNTR loci, STTR3, STTR5, and STTR9 were present in all strains. Analysis of loci diversity revealed that the STTR5 locus had the highest number of alleles (n = 12), whereas STTR6 and STTR9 showed the lowest diversity (Nei's diversity index of 0.34 and 0.26, respectively). VNTR loci STTR6 and STTR10pl were absent in most of the strains tested, with a typability of only 19.0% and 23.8%, respectively (Table 1).

Next, an allelic profile was constructed for each strain. The allelic profile was a string of 5 numbers, each indicating the number of repeat units in an individual locus (except STTR3), arranged in the order of STTR9-STTR5-STTR6-STTR10pl-STTR3. The number 00 indicates the absence of a specific VNTR locus.

Table 1. VNTR loci characteristics

Locus	No. of alleles	Allelic diversity[1)]	No. of strains	Typability (%)
STTR3	5	0.41	84	100.0
STTR5	12	0.70	84	100.0
STTR6	9	0.34	16	19.0
STTR9	7	0.26	84	100.0
STTR10pl	9	0.41	20	23.8

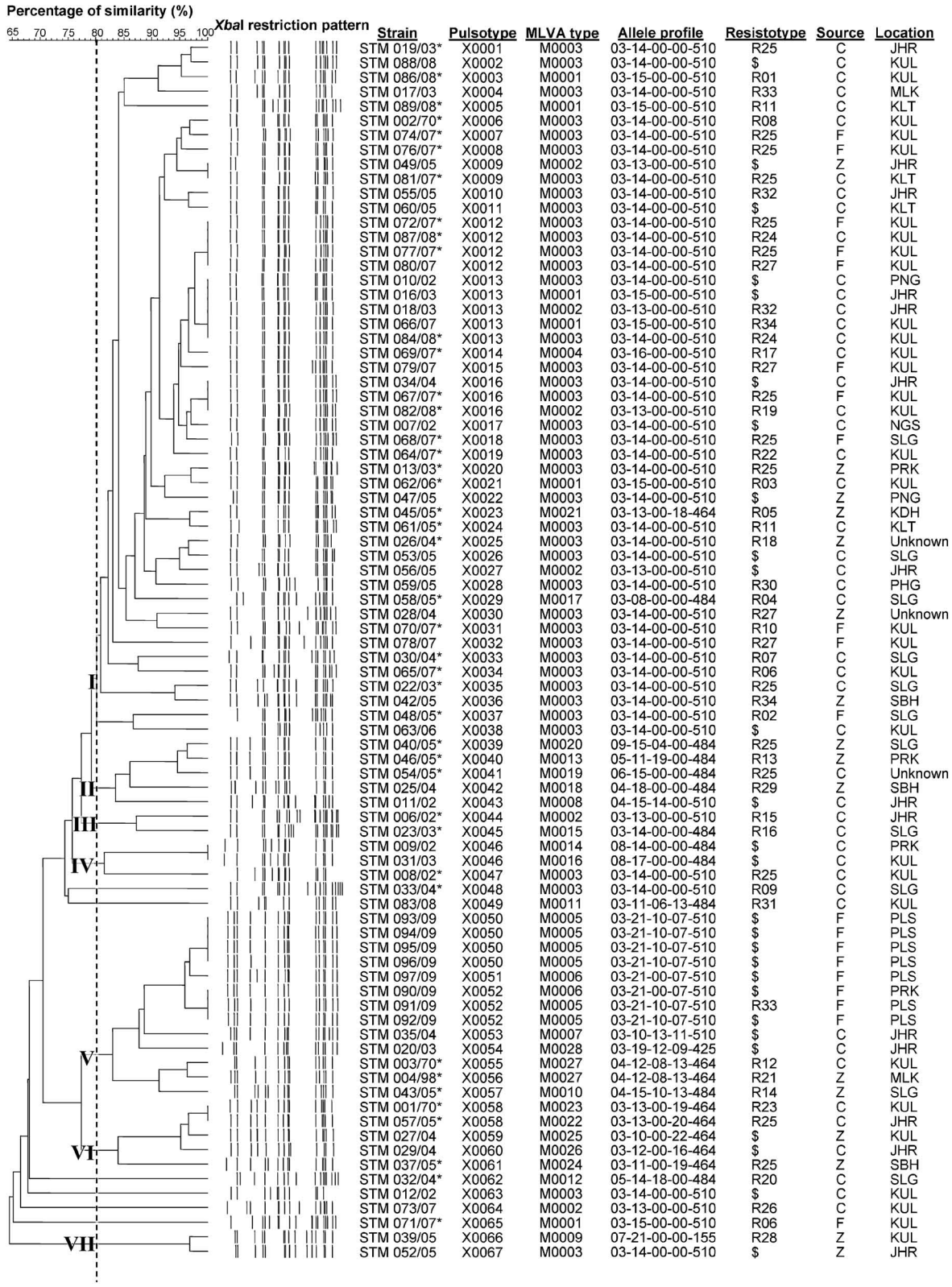
[1)]: Allelic diversity was calculated as $1 - \Sigma(\text{allele frequency})^2$ (Nei's diversity index).

The STTR3 locus contained 2 repeat units (27 and 33 bp, respectively); therefore, the actual amplicon length was used to construct an allele string according to the system adopted by Australian laboratories (18).

Altogether, 28 different allelic profiles (MLVA types) were identified (Fig. 1), and were arbitrarily designated as M0001 to M0028. The discriminatory power of MLVA was 0.76 (Simpson's diversity index). M0003 (allele string 03-14-00-00-510) was the most commonly found MLVA type (48%) among clinical (n = 23), zoonotic (n = 6), and food (n = 11) isolates (Fig. 1). MLVA types M0001 (03-15-00-00-510), M0002 (03-13-00-00-510), and M0003 (03-14-00-00-510) were single-locus variants (SLVs, varied in the STTR5 locus) that predominated (62%) in our strain collection (Fig. 1). Surprisingly, we observed little genetic variation among strains from different sources isolated years apart. For instance, a clinical strain (STM002/70) from 1970 shared identical MLVA type with strains as recent as 2008 (STM084/08, STM087/08, and STM088/08). When we studied the genetic relatedness of our strains using the MST method based on the allelic diversity of all the 5 VNTR loci, the strains were distributed throughout the tree with no clear branching dominated by strains from a single source, location, or year of isolation (Fig. 2).

Both STTR6 and STTR10pl loci were largely absent in the *S.* Typhimurium strains in this study. Therefore, DNA hybridization was performed on a selected subset of the strains to confirm the presence or absence of these 2 VNTR loci. A total of 23 *S.* Typhimurium strains with 20 strains positive for the STTR10pl locus were hybridized with a DIG-labeled STTR10pl probe. The result of hybridization concurred with the MLVA results, i.e., probe-target hybrids were detected for strains positive for the STTR10pl locus, while no signal was detected for strains negative for the STTR10pl locus (data not shown). Similarly, DNA hybridization using the DIG-labeled STTR6 probe for a selected subset of 23 strains with 6 strains positive for the STTR6 locus showed positive signals only for strains with STTR6.

PFGE analysis of *S.* Typhimurium strains: PFGE analysis of *Xba*I-digested chromosomal DNA from 84 *S.* Typhimurium strains yielded 67 pulsotypes, which were arbitrarily designated as X0001 to X0067 (Fig. 1). Each pulsotype contained 12 to 20 DNA fragments, and their sizes ranged from 20.5 to 1,135 kb. The genetic diversity (F value) of the strains ranged from 0.6 to 1.0, as determined by the PFGE pattern similarity. X0009 (n = 2), X0012 (n = 4), X0013 (n = 5), X0016 (n = 3), X0046 (n = 2), X0050 (n = 4), X0052 (n = 3), and



Strain	Pulsotype	MLVA type	Allele profile	Resistotype	Source	Location
STM 019/03*	X0001	M0003	03-14-00-00-510	R25	C	JHR
STM 088/08	X0002	M0003	03-14-00-00-510	$	C	KUL
STM 086/08*	X0003	M0001	03-15-00-00-510	R01	C	KUL
STM 017/03	X0004	M0003	03-14-00-00-510	R33	C	MLK
STM 089/08*	X0005	M0001	03-15-00-00-510	R11	C	KLT
STM 002/70*	X0006	M0003	03-14-00-00-510	R08	C	KUL
STM 074/07*	X0007	M0003	03-14-00-00-510	R25	F	KUL
STM 076/07*	X0008	M0003	03-14-00-00-510	R25	F	KUL
STM 049/05	X0009	M0002	03-13-00-00-510	$	Z	JHR
STM 081/07*	X0009	M0003	03-14-00-00-510	R25	C	KLT
STM 055/05	X0010	M0003	03-14-00-00-510	R32	C	JHR
STM 060/05	X0011	M0003	03-14-00-00-510	$	C	KLT
STM 072/07*	X0012	M0003	03-14-00-00-510	R25	F	KUL
STM 087/08*	X0012	M0003	03-14-00-00-510	R24	C	KUL
STM 077/07*	X0012	M0003	03-14-00-00-510	R25	F	KUL
STM 080/07	X0012	M0003	03-14-00-00-510	R27	F	KUL
STM 010/02	X0013	M0003	03-14-00-00-510	$	C	PNG
STM 016/03	X0013	M0001	03-15-00-00-510	$	C	JHR
STM 018/03	X0013	M0002	03-13-00-00-510	R32	C	JHR
STM 066/07	X0013	M0001	03-15-00-00-510	R34	C	KUL
STM 084/08*	X0013	M0003	03-14-00-00-510	R24	C	KUL
STM 069/07*	X0014	M0004	03-16-00-00-510	R17	C	KUL
STM 079/07	X0015	M0003	03-14-00-00-510	R27	F	KUL
STM 034/04	X0016	M0003	03-14-00-00-510	$	C	JHR
STM 067/07*	X0016	M0003	03-14-00-00-510	R25	F	KUL
STM 082/08*	X0016	M0002	03-13-00-00-510	R19	C	KUL
STM 007/02	X0017	M0003	03-14-00-00-510	$	C	NGS
STM 068/07*	X0018	M0003	03-14-00-00-510	R25	F	SLG
STM 064/07*	X0019	M0003	03-14-00-00-510	R22	C	KUL
STM 013/03*	X0020	M0003	03-14-00-00-510	R25	Z	PRK
STM 062/06*	X0021	M0001	03-15-00-00-510	R03	C	KUL
STM 047/05	X0022	M0003	03-14-00-00-510	$	Z	PNG
STM 045/05*	X0023	M0021	03-13-00-18-464	R05	Z	KDH
STM 061/05*	X0024	M0003	03-14-00-00-510	R11	C	KLT
STM 026/04*	X0025	M0003	03-14-00-00-510	R18	Z	Unknown
STM 053/05	X0026	M0003	03-14-00-00-510	$	C	SLG
STM 056/05	X0027	M0002	03-13-00-00-510	$	C	JHR
STM 059/05	X0028	M0003	03-14-00-00-510	R30	C	PHG
STM 058/05*	X0029	M0017	03-08-00-00-484	R04	C	SLG
STM 028/04	X0030	M0003	03-14-00-00-510	R27	Z	Unknown
STM 070/07*	X0031	M0003	03-14-00-00-510	R10	F	KUL
STM 078/07	X0032	M0003	03-14-00-00-510	R27	F	KUL
STM 030/04*	X0033	M0003	03-14-00-00-510	R07	C	SLG
STM 065/07*	X0034	M0003	03-14-00-00-510	R06	C	KUL
STM 022/03*	X0035	M0003	03-14-00-00-510	R25	C	SLG
STM 042/05	X0036	M0003	03-14-00-00-510	R34	Z	SBH
STM 048/05*	X0037	M0003	03-14-00-00-510	R02	F	SLG
STM 063/06	X0038	M0003	03-14-00-00-510	$	C	KUL
STM 040/05*	X0039	M0020	09-15-04-00-484	R25	Z	SLG
STM 046/05*	X0040	M0013	05-11-19-00-484	R13	Z	PRK
STM 054/05*	X0041	M0019	06-15-00-00-484	R25	C	Unknown
STM 025/04	X0042	M0018	04-18-00-00-484	R29	Z	SBH
STM 011/02	X0043	M0008	04-15-14-00-510	$	C	JHR
STM 006/02*	X0044	M0002	03-13-00-00-510	R15	C	JHR
STM 023/03*	X0045	M0015	03-14-00-00-484	R16	C	SLG
STM 009/02	X0046	M0014	08-14-00-00-484	$	C	PRK
STM 031/03	X0046	M0016	08-17-00-00-484	$	C	KUL
STM 008/02*	X0047	M0003	03-14-00-00-510	R25	C	KUL
STM 033/04*	X0048	M0003	03-14-00-00-510	R09	C	SLG
STM 083/08	X0049	M0011	03-11-06-13-484	R31	C	KUL
STM 093/09	X0050	M0005	03-21-10-07-510	$	F	PLS
STM 094/09	X0050	M0005	03-21-10-07-510	$	F	PLS
STM 095/09	X0050	M0005	03-21-10-07-510	$	F	PLS
STM 096/09	X0050	M0005	03-21-10-07-510	$	F	PLS
STM 097/09	X0051	M0006	03-21-00-07-510	$	F	PLS
STM 090/09	X0052	M0006	03-21-00-07-510	$	F	PRK
STM 091/09	X0052	M0005	03-21-10-07-510	R33	F	PLS
STM 092/09	X0052	M0005	03-21-10-07-510	$	F	PLS
STM 035/04	X0053	M0007	03-10-13-11-510	$	C	JHR
STM 020/03	X0054	M0028	03-19-12-09-425	$	C	JHR
STM 003/70*	X0055	M0027	04-12-08-13-464	R12	C	KUL
STM 004/98*	X0056	M0027	04-12-08-13-464	R21	Z	MLK
STM 043/05*	X0057	M0010	04-15-10-13-484	R14	Z	SLG
STM 001/70*	X0058	M0023	03-13-00-19-464	R23	C	KUL
STM 057/05*	X0058	M0022	03-13-00-20-464	R25	C	JHR
STM 027/04	X0059	M0025	03-10-00-22-464	$	Z	KUL
STM 029/04	X0060	M0026	03-12-00-16-464	$	C	JHR
STM 037/05*	X0061	M0024	03-11-00-19-464	R25	Z	SBH
STM 032/04*	X0062	M0012	05-14-18-00-484	R20	C	SLG
STM 012/02	X0063	M0003	03-14-00-00-510	$	C	KUL
STM 073/07	X0064	M0002	03-13-00-00-510	R26	C	KUL
STM 071/07*	X0065	M0001	03-15-00-00-510	R06	F	KUL
STM 039/05	X0066	M0009	07-21-00-00-155	R28	Z	KUL
STM 052/05	X0067	M0003	03-14-00-00-510	$	Z	JHR

Fig. 1. Dendrogram showing a cluster analysis of 84 *Salmonella* Typhimurium strains based on the PFGE profiles of the *Xba*I-digested chromosomal DNA of the bacterial strains. The dendrogram was constructed using the Dice coefficient (*F*) and UPGMA clustering parameters at 1.5% position tolerance. Roman numerals I to VII denote strain clusters with a cutoff value of $F = 0.80$. Strains marked with an asterisk (*) are multidrug resistant. The year of isolation is indicated as the last 2 digits of the strain code (e.g., STM001/70 was isolated in 1970). The allelic profile indicates the allele numbers of the VNTR loci (except STTR3, where the number indicates the amplicon length), arranged in the order STTR9-STTR5-STTR6-STTR10pl-STTR3. The number 00 indicates that no amplicon was obtained. The resistotypes of the strains correspond with data shown in Table 2. The sources of the strains are indicated as letters, where ''C'' represents clinical strains, ''Z'' represents zoonotic strains, and ''F'' represents food strains. JHR, Johor; KDH, Kedah; KLT, Kelantan; KUL, Kuala Lumpur; MLK, Melaka; NGS, Negeri Sembilan; PHG, Pahang; PLS, Perlis; PNG, Penang; PRK, Perak; SBH, Sabah; SLG, Selangor.

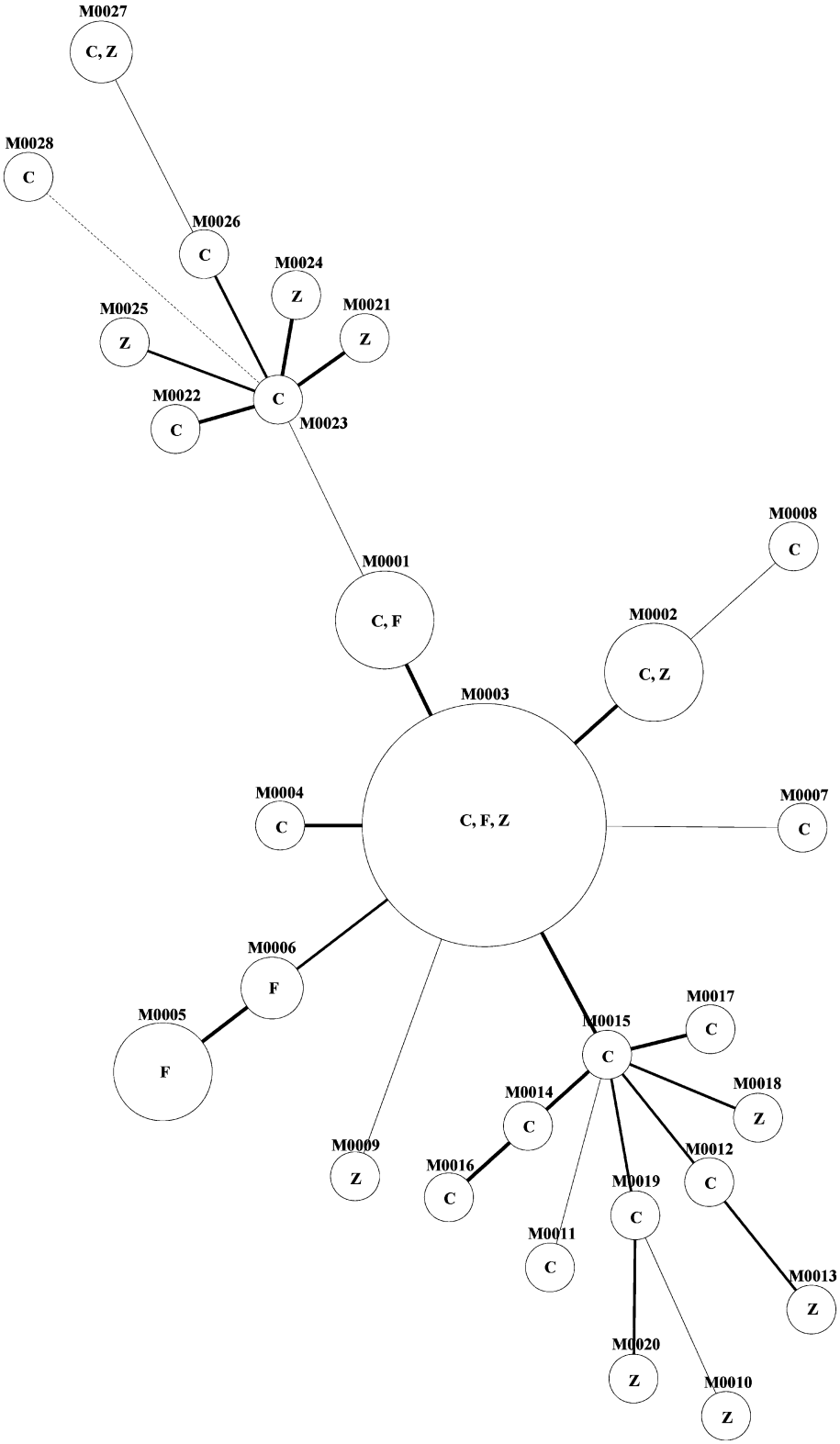


Fig. 2. Minimum spanning tree for MLVA of 84 *S.* Typhimurium strains. A circle denotes an MLVA type. The thickness and dotting of the lines indicated the allelic differences between the MLVA types; thus, a thick line indicates a single-locus variant, a thin line indicates allelic differences at 2 different VNTR loci, and a dotted line denotes allelic differences at 3 or more VNTR loci. The sources of the strains are indicated as letters in the circles, where ''C'' represents clinical strains, ''Z'' represents zoonotic strains, ''F'' represents food strains, ''C, Z'' represents a mix of clinical and zoonotic strains, ''C, F'' represents a mix of clinical and food strains, and ''C, Z, F''' represents a mix of strains from all 3 sources.

X0058 (n = 2) were common pulsotypes that appeared in more than 1 strain. The remaining 60 pulsotypes were unique. The Simpson's index of diversity for PFGE was 0.99.

A dendrogram based on 67 pulsotypes revealed 7 clusters (clusters I to VII) of closely related strains, with a Dice coefficient of similarity of 0.80 (Fig. 1). Cluster I (F = 0.80) was the major group, consisting 57% (n = 48) of the studied strains. The majority of the strains in this cluster were from clinical sources (n = 30), followed by food (n = 11) and zoonotic (n = 7) sources, and were isolated between 1970 and 2008. Within this cluster, 44% of the strains were isolated from the capital city Kuala Lumpur (n = 21). Clusters II (F = 0.83), III (F = 0.87), IV (F = 0.81), VI (F = 0.83), and VII (F = 0.96) were smaller and contained 2 to 5 strains.

Antimicrobial drug susceptibility of *S.* Typhimurium strains: Of the 84 *S.* Typhimurium strains screened, 27 strains were susceptible to all the 16 antimicrobial agents tested (Table 2). We observed high rates of resistance to AMP (25%), CEF (26%), SUL (49%), TET (51%), and STR (52%). The presence of MDR *S.* Typhimurium strains (defined as strains resistant to 3 or more different classes of antimicrobial agents) in our strain collection was as high as 50%. These included strains that were isolated from 1970 to 2008 from all sources. The MDR strains mostly originated from urban and densely populated areas such as Kuala Lumpur and Selangor.

A total of 34 resistotypes were identified from our screening and were arbitrarily designated as R01 to R34 (Table 2). Resistotype R25 was the most dominant, showing simultaneous resistance to STR, SUL, and TET (R-type SSuT). Overall, the *S.* Typhimurium strains in our study showed high susceptibility to third-generation cephalosporins. Less than 5% of the strains tested were resistant to CTX, CAZ, and CRO (Table 2). Quinolone resistance was observed; specifically, 20% of our samples were resistant to NAL, CIP, or both.

Determination of *S.* Typhimurium flagellar phase:

Table 2. Antimicrobial susceptibility profiles of *S.* Typhimurium strains

Resistotype	Resistance profile[1)]																No. of isolates
R01	AMC	AMP	CEF	—	CAZ	—	CHL	—	NAL	GEN	KAN	STR	TET	SXT	SUL	—	1
R02	AMC	AMP	CEF	—	—	—	CHL	—	NAL	—	KAN	STR	TET	SXT	SUL	TMP	1
R03	—	AMP	—	—	—	—	CHL	CIP	NAL	GEN	KAN	STR	TET	SXT	SUL	TMP	1
R04	AMC	AMP	CEF	CTX	CAZ	CRO	—	CIP	—	—	—	STR	TET	—	SUL	—	1
R05	—	AMP	CEF	—	—	—	CHL	—	NAL	—	KAN	STR	TET	SXT	SUL	TMP	1
R06	—	AMP	CEF	—	—	—	CHL	—	NAL	GEN	KAN	STR	TET	SXT	SUL	—	2
R07	AMC	AMP	CEF	CTX	CAZ	CRO	CHL	—	—	—	—	—	—	—	SUL	—	1
R08	AMC	AMP	CEF	—	—	—	CHL	—	—	—	KAN	STR	TET	—	SUL	—	1
R09	—	AMP	CEF	CTX	CAZ	CRO	—	—	—	—	—	STR	TET	—	SUL	—	1
R10	—	AMP	CEF	—	—	—	CHL	—	—	—	KAN	STR	TET	SXT	SUL	—	1
R11	—	AMP	—	—	—	—	CHL	—	—	—	KAN	STR	TET	SXT	SUL	TMP	2
R12	AMC	AMP	CEF	—	—	—	CHL	—	—	—	KAN	—	TET	—	SUL	—	1
R13	AMC	—	CEF	—	—	—	—	—	—	—	KAN	STR	TET	—	SUL	TMP	1
R14	—	AMP	—	—	—	—	—	—	NAL	—	—	STR	TET	SXT	SUL	TMP	1
R15	—	AMP	CEF	—	—	—	—	—	—	—	—	STR	TET	SXT	SUL	TMP	1
R16	—	AMP	—	—	—	—	CHL	—	—	—	—	STR	—	SXT	SUL	TMP	1
R17	—	—	CEF	—	—	—	—	—	—	—	—	STR	TET	SXT	SUL	TMP	1
R18	AMC	AMP	CEF	—	—	—	—	—	NAL	—	—	—	—	—	SUL	—	1
R19	—	—	CEF	—	—	—	—	—	—	—	—	STR	TET	—	SUL	TMP	1
R20	—	AMP	—	—	—	—	—	—	NAL	GEN	—	—	TET	—	—	—	1
R21	—	—	—	—	—	—	—	—	NAL	GEN	—	STR	TET	—	—	—	1
R22	—	—	CEF	—	—	—	—	—	NAL	—	—	STR	TET	—	—	—	1
R23	—	—	CEF	—	—	—	—	—	—	—	—	STR	TET	—	SUL	—	1
R24	—	—	CEF	—	—	—	—	—	—	—	—	STR	TET	—	—	—	2
R25	—	—	—	—	—	—	—	—	—	—	—	STR	TET	—	SUL	—	15
R26	—	—	CEF	—	—	—	—	—	NAL	—	—	—	—	—	—	—	1
R27	—	—	—	—	—	—	—	—	—	—	—	STR	—	—	SUL	—	4
R28	—	—	—	—	—	—	—	—	NAL	—	—	—	TET	—	—	—	1
R29	—	—	—	—	—	—	—	—	—	—	—	—	TET	—	SUL	—	1
R30	—	AMP	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1
R31	—	—	CEF	—	—	—	—	—	—	—	—	—	—	—	—	—	1
R32	—	—	—	—	—	—	—	—	NAL	—	—	—	—	—	—	—	2
R33	—	—	—	—	—	—	—	—	—	—	—	STR	—	—	—	—	2
R34	—	—	—	—	—	—	—	—	—	—	—	—	TET	—	—	—	2
$	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	27

[1)]: The resistance profiles excluded antimicrobial agents that exhibited intermediate resistance.
AMC, amoxicillin-clavulanic acid; AMP, ampicillin; CEF, cephalothin; CTX, cefotaxime; CIP, ciprofloxacin; CAZ, ceftazidime; CHL, chloramphenicol; CRO, ceftriaxone; GEN, gentamicin; KAN, kanamycin; STR, streptomycin; TET, tetracycline; SXT, trimethoprim-sulfamethoxazole; NAL, nalidixic acid; SUL, compound sulfonamide; TMP, trimethoprim; $, sensitive to all antimicrobial agents tested.

The majority of our samples (99%, $n = 83$) were biphasic *S.* Typhimurium. Only 1 strain (STM032/04) of clinical origin isolated in 2004 was identified as a monophasic variant of *S.* Typhimurium, lacking the *fljB* H:1,2 allele.

DISCUSSION

S. Typhimurium infection is a common foodborne infection in developing countries; however, there is a lack of systematic characterization of existing strains in many parts of Southeast Asia. We report here the first MLVA-based subtyping of *S.* Typhimurium strains in Malaysia. Nei's diversity index indicated that the diversity indices for STTR5, STTR6, STTR9, and STTR10pl were lower in our sample population as compared to the values reported by Lindstedt et al. (15). The absence of STTR6 and STTR10pl in most strains (81.0% and 76.2%, respectively) further contributed to the low diversity. To rule out the possibility of false-negative results for these 2 loci, we performed DNA hybridization, and confirmed that STTR6 and STTR10pl were indeed absent (data not shown). The low prevalence of these loci may be attributed to the association of STTR6 with Gifsy–1 prophage and of STTR10pl with the plasmid pSLT (15). We postulated that most Malaysian *S.* Typhimurium strains lacked these 2 genetic entities; future studies are needed to elucidate this finding. There seems to be a difference between Asian and European strains. Notably, 30% of the strains obtained from Southeast Asia (Thailand, Malaysia, and Indonesia) were negative for STTR6 and 97% were negative for STTR10pl (B. A. Lindstedt, personal communication). In contrast, only 16% and 48% of the strains from Norway were negative for STTR6 and STTR10pl, respectively (15). On the contrary, STTR3 showed higher diversity in our samples. Nevertheless, the overall low diversity indices (0.26–0.70) for all 5 VNTR loci suggested that these loci are less discriminative if used as molecular markers for subtyping *S.* Typhimurium in this geographical region. The inclusion of other VNTR loci that are yet to be examined may prove useful for more precise strain mapping.

By comparing the MLVA and PFGE methods using the same strains, we found that MLVA subtyping was less discriminative ($D = 0.76$) than PFGE ($D = 0.99$); nevertheless, we still found a strong correlation. For example, the PFGE cluster I coincided with the SLVs M0001, M0002, M0003, and M0004, while M0005 and M0006 (SLVs) corresponded with the PFGE cluster V. Both typing methods suggested a rather homogeneous *S.* Typhimurium population circulated within Malaysia during this period of study. The MST constructed (Fig. 2) did not show clear branching of strains exclusively by a single source, location, or year of isolation in the overall distribution of the MLVA types. Because MLVA is reported to be sensitive to the origins of the strains (15), this observation further supports the inference regarding the genetic homogeneity of local *S.* Typhimurium strains in Malaysia. This should probably be expected, as nontyphoidal salmonellosis caused by *S.* Typhimurium is endemic in this region. The close genetic relationship between clinical, food, and zoonotic strains was seen in the sharing of similar allelic profiles and pulsotypes among the strains. This suggested that the *S.* Typhimurium from farm animals might infect humans, with contaminated food serving as the vehicle.

Overall, in this study, we observed a high percentage (50%) of MDR *S.* Typhimurium strains isolated from various sources and locations. All earlier strains (isolated in 1970 and 1998) showed multidrug resistance. However, because of the 30-year gap in the years of isolation, we could not conclude whether the MDR phenotypes had persisted since 1970 or whether there was a relapse between 1970 and 2002. More strains isolated between these 2 years should be included to provide a better understanding of the prevalence of MDR phenotypes in Malaysia over the years.

Previous reports on the occurrence and characterization of MDR *S.* Typhimurium were source-specific (2,3). Therefore, the present study provides a more extensive analysis, by encompassing strains from clinical, zoonotic, and food sources. The food-derived and clinically-derived MDR strains mostly originated from developed and densely populated areas such as Kuala Lumpur and Selangor. The high genetic proximity among these strains suggested the probable spread of the pathogen from food to humans. This phenomenon is not uncommon in Malaysia, because the isolation of pathogenic *Salmonella* serovars from RTE food was frequently reported (2). Additionally, half of the zoonotic strains screened in this study showed MDR phenotypes, suggesting that the occurrence of MDR strains in food, especially meat and poultry products, might be due to the use of antimicrobial agents in animal feeds at livestock farms. Previous studies showed that food animals indeed served as a reservoir of MDR *Salmonella* (29–31).

The most common type of MDR *S.* Typhimurium strain in Malaysia was R-type SSuT (27.4%), followed by a hexa-resistant pattern R-type ACKSSuT (11.9%; strains showed simultaneous resistance to AMP, CHL, KAN, STR, SUL, and TET). The resistance to these conventional antimicrobial agents has also been reported previously (32–34). Mixed results were obtained for resistance to newer antimicrobial agents such as quinolone; resistance to NAL was shown by a relatively high number of strains (18%); however, resistance to CIP was rare (2%). Resistance to third-generation cephalosporins was very low in our study (CTX, $n = 3$; CAZ, $n = 4$; and CRO, $n = 3$). These findings indicated that the newer drugs remained effective in this region during the study period. However, we should also be wary of the emergence of resistant strains, although the frequency is low. More recent strains should be included in future studies so as to accurately reflect the antimicrobial resistance of currently circulating *S.* Typhimurium populations in Malaysia.

The worldwide emergence and spreading of monophasic *Salmonella* 4,[5],12:i:– has been reported since 1990s (8). In Asia, the isolation of this clinically important NTS from clinical samples has been reported in Thailand and Taiwan (35,36) but not in Malaysia. In this study, we found only 1 monophasic variant of *S.* Typhimurium (STM032/04) from a hospitalized patient in 2004. It has been known that the majority of *Salmonella* 4,[5],12:i:– strains reported in European and Asian countries showed MDR phenotypes (8). We

found that the monophasic STM032/04 strain in this study was resistant to AMP, GEN, and TET, in addition to NAL; this finding was similar to those obtained for monophasic strains in Thailand (37). The PCR detection of a serotype Typhimurium-specific sequence (22) adopted in this study failed to distinguish between monophasic strains and their biphasic counterparts. In a nutshell, these findings indicated that the monophasic *Salmonella* 4,[5],12:i:– is relatively uncommon in this region, and so far, there have been no reports of an outbreak caused by this serovar in Malaysia. However, we acknowledge a caveat here, which is that *Salmonella* 4,[5],12:i:– is commonly misclassified as *S.* Typhimurium by both conventional serotyping and PCR detection methods, and this could lead to underreporting of cases. A larger sample size and more careful analysis in the future are needed to determine and monitor the spreading of this strain.

To our knowledge, this study represents the first detailed report of the molecular characteristics of *S.* Typhimurium strains in Malaysia, studying the genotypic and phenotypic relationships among the strains through MLVA and PFGE subtyping and determining the antimicrobial resistance patterns and flagellar phases of the strains. We conclude that the MLVA and PFGE typing of the *S.* Typhimurium strains showed moderate congruency in strain clustering, but PFGE ($D = 0.99$) was more discriminative than MLVA (0.76) on the basis of the 5 VNTR loci screened. A multiple-typing approach encompassing the gold standard PFGE and the high-throughput MLVA assays for detailed strain differentiation is suggested for future epidemiological study of the pathogen. Finally, in this study, the biphasic *S.* Typhimurium strains were dominant, and most of these strains showed MDR phenotypes.

Acknowledgments This study was funded by University of Malaya Post Graduate Research Fund (PPP) (PS319/2010B), University of Malaya Research Grant (RG017-09BIO), and Japanese National Institute of Infectious Diseases Grant (57-02-03-1009).

We thank Dr Ser Huy Teh and Dr Cindy Shuan Ju Teh for their technical assistance.

Conflict of interest None to declare.

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