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Characterization of Drug-Resistant *Salmonella enterica* Serotype Typhimurium by Antibiograms, Plasmids, Integrons, Resistance Genes, and PFGE

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Forty-seven *Salmonella* Typhimurium (33 zoonotic, 14 clinical) strains were tested for antimicrobial resistance using the standard disk diffusion method. The presence of relevant resistance genes and class 1 integrons were investigated by using PCR. Pulsed-field gel electrophoresis (PFGE) and plasmid profiling were carried out to determine the genomic diversity of *Salmonella* Typhimurium. Approximately 57.4% of the *S.* Typhimurium strains were multidrug resistant (MDR) and showed high resistance rates to tetracycline (70.2%), sulfonamides (57.4%), streptomycin (53.1%), ampicillin (29.7%), nalidixic acid (27.6%), kanamycin (23.4%), chloramphenicol (21.2%), and trimethoprim (19.1%). Resistance towards cephalosporins was noted for cephalothin (27.6%), cephradine (21.2%), amoxicillin clavulanic acid (17.0%), and cephalexin (17.0%). Resistance genes, *bla*$_{TEM}$, *strA*, *aadA*, *sul1*, *sul2*, *tetA*, *tetB*, and *tetC*, were detected among the drug-resistant strains. Thirty-three strains (70.2%) carried class 1 integrons, which were grouped in 9 different profiles. DNA sequencing identified *sat*, *aadA*, *pse-1*, and *dfrA* genes in variable regions on class 1 integrons. Thirty-five strains (74.4%) were subtyped to 22 different plasmid profiles, each with 1–6 plasmids (2.0 to 95 kb). PFGE subtyped the 47 strains into 39 profiles. In conclusion, high rates of multidrug resistance were found among the Malaysian *Salmonella* Typhimurium strains. The emergence of multidrug-resistant *Salmonella* Typhimurium to cephalosporin antibiotics was also observed. The strains were very diverse and no persistent clone was observed. The emergence of MDR *Salmonella* Typhimurium is a worldwide problem, and this report provides information for the better understanding of the prevalence and epidemiology of MDR *S.* Typhimurium in Malaysia.

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Human salmonellosis continues to be a major public health problem in both developed and developing countries. Nontyphoidal salmonellosis (NTS), caused by salmonellae other than *Salmonella* Typhi, is by far the most common cause of bacteremia associated with gastroenteritis. Between 1973 and 1982, *Salmonella* Typhimurium was the most common *Salmonella* isolated from patients attending the University Hospital in Kuala Lumpur, Malaysia. Out of a total of 969 strains isolated from blood and feces (excluding *S.* Typhi), 155 (~16%) were *Salmonella* Typhimurium (S.D. Puthucheary, personal communication). *Salmonella* Typhimurium was also the most common NTS (10%) isolated throughout Malaysia during the same decade [34] but dropped to 4.06% from 1983 to 1992.

The occurrence of *Salmonella* Typhimurium phage type 104 (DT104) resistant to ampicillin, chloramphenicol, streptomycin, sulfonamide, and tetracycline has been reported worldwide [29]. The resistance of this serovar to cephalosporins, due to the production of extended-spectrum β-lactamase (ESBL), has also been reported [26]. Dissemination of the antimicrobial resistance is often through mobile genetic elements such as plasmids, transposons, and gene cassettes in integrons [20]. Class 1 integrons are most frequently found among clinical isolates that contain antimicrobial resistance genes in the internal variable region. This integron-containing antibiotic resistance gene cluster has been found near the 3' end of *Salmonella* genomic island 1 (SGI1) of serovar Typhimurium [8].

Phenotypic methods for identifying and typing of *Salmonella* Typhimurium are serotyping, biotyping, and phage typing, all showing limited discriminatory powers. Plasmid profiling, ribotyping, IS200 typing, multilocus

sequence typing (MLST), PCR-based methods, and pulsed-field gel electrophoresis (PFGE) have recently proven to be useful in discriminating strains of *S.* Typhimurium [13, 32].

There is a lack of information on *S.* Typhimurium in Malaysia and this study was carried out to obtain better strain discrimination of this serovar isolated between 1969 to 2006 from animals and humans through a combination of methods. The detection and analysis of antibiotic resistance genes and integrons were carried out by using PCR and DNA sequencing, and the genetic relatedness was determined by PFGE and plasmid fingerprinting.

Materials and Methods

Bacterial Strains

Forty-seven *Salmonella* Typhimurium strains were studied. The human strains (n=14) isolated from 1969 to 2006 were from a local teaching hospital. The zoonotic strains (n=33) recovered from a variety of animals (cattle, n=9; chicken, n=14; swine, n=1; fish, n=1; frog, n=1) and unknown zoonotic hosts (n=7) were provided by the Veterinary Research Institute, Malaysia. All the strains were confirmed to be *S.* Typhimurium by standard biochemical and serological tests at the Microbiology Laboratories of the Institutions that processed the specimens. The purity of the strains was checked by plating the cultures on selective media (XLD and BSA) at the Biomedical Science Laboratory, IPS, University of Malaya, where the analysis was carried out.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was done using the disk diffusion assay on Mueller–Hinton agar with commercial antibiotic disks (Oxoid Ltd, Basingstoke, U.K.) according to the CLSI guidelines [11]. *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as controls. The antimicrobials used were nalidixic acid (10 μg), ampicillin (10 μg), streptomycin (10 μg), ciprofloxacin (5 μg), trimethoprim/sulfamethoxazole (25 μg), tetracycline (30 μg), chloramphenicol (30 μg), trimethoprim (5 μg), gentamicin (30 μg), kanamycin (30 μg), sulfonamide (300 μg), amoxicillin/clavulanic acid (30 μg), ceftiofur (30 μg), cefepime (30 μg), aztreonam (30 μg), cephalothin (30 μg), ceftazidime (30 μg), cephradine (30 μg), ceftriaxone (30 μg), cephalexin (30 μg), cefuroxime (30 μg), and cefotaxime (30 μg) (Becton Dickinson). Interpretation of inhibition zones was according to CLSI guidelines [11]. Interpretations for ceftiofur, which was not given in the CLSI guidelines, was as recommended by the manufacturer.

DNA Template Preparation for PCR

One bacterial colony from Luria–Bertani (LB) plate was suspended in 1.0 ml of phosphate-buffered saline (PBS), centrifuged, and the cell pellet resuspended in 100 μl of TE buffer (10 mM Tris, 1 mM EDTA buffer, pH 8.0) and boiled at 99°C for 10 min. For each PCR reaction, 5 μl (~100 ng) of the supernatant was used.

Differentiation of Phage Type DT104

As phage typing facilities are not available in Malaysia, further differentiation of *S.* Typhimurium to phage type DT104, a commonly reported phage type associated with zoonotic strains, was performed using a multiplex PCR. Published primers for confirmation of *Salmonella* genus, *Salmonella* Typhimurium, and phage type DT104 are indicated in Table 1. PCR conditions consisted of an initial denaturation at 95°C for 2 min, 30 cycles each of 95°C for 1 min, 57°C for 1 min, and 72°C for 2 min, and a final extension at 72°C for 5 min [4].

Detection of Resistance Genes

PCR detection and confirmation of resistance to ampicillin, streptomycin, sulfonamides, and tetracycline were carried out using published primer sequences (Table 1). Initially, a monoplex-PCR was used for each of the primer sets to determine its specificity and reproducibility. Then, 3 multiplex-PCRs (mPCR) were optimized to enable simultaneous amplification of multiple resistance genes. mPCRI consisted of 4 primer pairs for bla_{TEM}, *strA*, *sul1*, and *sul2*. mPCRII consisted of another 4 primer pairs for *tetA*, *tetB*, *temB*, and *aadA*, whereas mPCRIII consisted of 3 primer pairs for *temA*, *tetG*, and *tetC*. The conditions for mPCRI were an initial denaturation at 94°C for 3 min followed by 30 cycles of 94°C for 30 s, 53°C for 30 s, and 72°C for 1 min, with a single final extension at 72°C for 7 min. For mPCRII and mPCRIII, the annealing conditions were changed to 62°C for 30 s. All mPCRs were performed in a 25-μl reaction mixture containing 1× PCR buffer, 1.5 mM $MgCl_2$, 200 μM each of dNTPs, 60 pmol of each primer, 1 U of *Taq* DNA polymerase (Intron, Biotechnology, South Korea), and 100 ng of template DNA. The PCR products were analyzed by electrophoresis through a 1.5% agarose gel (Promega, Madison, U.S.A), staining with ethidium bromide (0.5 μg/ml), and then visualizing using the GelDoc (BioRad). A DNA ladder of 100 bp (Promega) was used as the molecular weight marker.

Detection of Integrons

Isolates were screened for the presence of class 1 integrons using specific primers previously described [20] to anneal the 5' conserved sequences (CS) and 3' CS flanking the integrated gene cassettes (Table 1). PCR was performed in a 25-μl final volume containing 1× PCR buffer, 1.5 mM $MgCl_2$, 200 μM each of dNTPs, 1 U *Taq* DNA polymerase (Intron, Biotechhnology, South Korea), 25 pmol of each primer, and 100 ng of template DNA. The cycling conditions consisted of an initial denaturation at 94°C 10 min, 35 cycles each of 94°C for 1 min, 55°C for 1 min, and 72°C for 5 min, and further extension at 72°C for 5 min [12]. The amplification reaction included a negative control, which contained all reagents except target DNA. The PCR products were analyzed by electrophoresis on 1% agarose gel.

DNA Sequencing

Selected amplified DNA products were verified by DNA sequencing. The amplicons were purified using a DNA purification kit (Qiagen, Germany) and sent to a commercial facility for sequencing (Research Biolabs, Singapore). The ABI PRISM Big Dye terminator cycle sequencing system was used (Perkin Elmer, Applied Biosystems). The resulting DNA sequence data were compared with the GenBank database using the BLAST algorithm available on the NCBI Web site (http://www.ncbi.nih.gov).

Plasmid Profiling

Plasmid DNA was extracted using a Q1Aprep spin miniprep kit (Qiagen, Germany) according to the manufacturer's recommendation. The plasmid DNA was analyzed through 0.8% horizontal agarose

Table 1. Primers for detection of resistance genes and integrons in *Salmonella via* PCR.

Primers names	Sequence (5'–3')	Amplification target	Size (bp)	Reference
OMPCF OMPCR	ATCGCTGACTTATGCAATCG CGGGTTGCGTTATAGGTCGT	*Salmonella* genus	204	[4]
104F 104R	ATGCGTTTGGTCTCAACGCC GCTGAGGCCACGGATATTTA	*S.* Typhimurium DT104	102	[4]
TyphF TyphR	TTGTTCACTTTTTACCCCTGAA CCCTGACAGCCGTTAGATATT	*S.* Typhimurium	401	[4]
5'CS 3'CS	GGCATCCAAGCAGCAAG AAGCAGACTTGACCTGA	Integron	Variable	[20]
blaTem	ATGAGTATTCAACATTTCCG ACCAATGCTTAATCAGTGAG	bla_{TEM}	859	[2]
StrA	CCAATCGCAGATAGAAGGC CTTGGTGATAACGGCAATTC	*strA*	548	[2]
Sul1	CTTCGATGAGAGCCGGCGGC GCAAGGCGGAAACCCGCGCC	*sul1*	435	[2]
Sul2	GCGCTCAAGGCAGATGGCATT GCGTTTGATACCGGCACCCGT	*sul2*	293	[2]
temA	ATGAGTATTCAACATTTCCG CTGACAGTTACCAATGCTTA	*temA*	867	[23]
temB	TTTTCGTGTCGCCCTTATTCC CGTTCATCCATAGTTGCCTGACTC	*temB*	79	[31]
TetA	GTAATTCTGAGCACTGTCGC CTGCCTGGACAACATTGCTT	*tetA*	956	[2]
TetB	CTCAGTATTCCAAGCCTTTG ACTCCCCTGAGCTTGAGGGG	*tetB*	414	[2]
TetC	GGTTGAAGGCTCTCAAGGGC CCTCTTGCGGGAATCGTCC	*tetC*	505	[2]
TetG	GCAGCGAAAGCGTATTTGCG TCCGAAAGCTGTCCAAGCAT	*tetG*	662	[2]
aadA	ATCCTTCGGCGCGATTTTG GCAGCGCAATGACATTCTTG	*aadA*	282	[2]

gel electrophoresis. *E. coli* 39R and *E. coli* V517 were used as molecular weight markers.

PFGE Analysis

PFGE was carried out according to a previous protocol [28]. DNA was digested with 10 U of restriction enzyme *Xba*1 (5'-TCTAGA-3') (Promega, Madison, WI, U.S.A.) at 37°C. The restriction fragments were separated by electrophoresis in 0.5× TBE buffer, for 22 h at 14°C in a CHEF Mapper system (Bio-Rad, U.S.A.) using pulsed times of 2.2 to 63.8 s. *Xba*I-digested *S*. Braenderup H9812 was used as the DNA size marker. PFGE data were analyzed using Gel Compar software (version 4, Applied Maths). The extent of variability was determined by the Dice coefficient F, as previously described [28]. Clustering was based on the unweighted pair group average method (UPGMA) with a position tolerance of 0.10.

RESULTS

PCR and Phage types

The multiplex PCR products consisted of a 204-bp fragment of the *omp*C sequence that is specific for the *Salmonella* genus, a 401-bp fragment of the specific sequence in Typhimurium, and a 102-bp fragment of the specific sequence in DT104. Out of the initial 59 *S*. Typhimurium isolates provided by the collaborating centers, 47 were confirmed as serotype *Salmonella* Typhimurium as indicated by the presence of 2 bands, 204 bp and 401 bp. The other 12 *Salmonella* strains had only one amplicon, 204 bp. Among the 47 *S*. Typhimurium strains, 20 were phage type DT104.

Antibiograms

Only 9 of the 47 confirmed *S*. Typhimurium strains were susceptible to all the 22 antimicrobials tested, whereas 33 (70.2%) were resistant to more than one. All the animal strains were susceptible to ciprofloxacin, cefuroxime, ceftazidime, and aztreonam, whereas the human strains were resistant to at least one antimicrobial agent (Table 2). High rates of resistance to tetracycline (70.2%), sulfonamide (57.4%), streptomycin (53.1%), ampicillin (29.7%), nalidixic acid (27.6%), kanamycin (23.4%), chloramphenicol (21.2%), and trimethoprim (19.1%) were observed for both human and zoonotic strains (Table 2). Resistance towards cephalosporins

Table 2. Antimicrobial resistance among *S.* Typhimurium strains.

Antimicrobial agents	Numbers (%) of resistant strains		
	Animal (n=33)	Human (n=14)	Total
Ampicillin	8 (24)	6 (43)	14 (30)
Chloramphenicol	5 (15)	5 (36)	10 (21)
Ciprofloxacin	0 (0)	1 (7)	1 (2)
Gentamicin	4 (12)	1 (7)	5 (11)
Kanamycin	5 (15)	6 (43)	11 (23)
Streptomycin	15 (45)	10 (71)	25 (53)
Nalidixic acid	12 (36)	1 (7)	13 (28)
Sulfonamides	16 (48)	11 (79)	27 (57)
Trimethoprim	5 (15)	4 (29)	9 (19)
Sxt	4 (12)	2 (14)	6 (15)
Tetracycline	21 (64)	12 (86)	33 (70)
Cephradine	5 (15)	5 (36)	10 (21)
Cephalexin	2 (6)	6 (43)	8 (17)
Ceftriaxone	1 (3)	1 (7)	2 (4)
Cefuroxime	0 (0)	1 (7)	1 (2)
Cephalothin	7 (21)	6 (43)	13 (28)
Ceftazidime	0 (0)	1 (75)	1 (2)
AMC	4 (12)	4 (29)	8 (17)
Cefepime	0 (0)	1 (7)	1 (2)
Cefotaxime	1 (3)	1 (7)	2 (5)
Aztreonam	0 (0)	1 (7)	1 (2)
Ceftiofur	0 (0)	1 (7)	1 (2)

Sxt, trimethoprim/sulfamethoxazole; AMC, amoxicillin–clavulanic acid.

was as follows: cephalothin (27.6%), cephradine (21.2%), amoxicillin (17.0%), and cephalexin (17.0%). Twenty-nine resistotypes were defined (Table 3). The predominant resistotype was P8 (simultaneous resistance to S, Su, T), followed by resistotypes P3 and P7. The rest of the resistotypes were represented by one strain each (Table 3).

Multidrug resistance (MDR), defined as resistance to 3 or more groups of antimicrobial agents, was detected in 27 (57.4%) strains (Table 3). Three common R-types were observed: R-type ACSSuTe (n=8), R-type SSuTe (n=7), and R-type KSSuTeTm (n=3) with or without additional resistance to antimicrobial agents.

Detection of Resistance Genes

The simultaneous detection of different antimicrobial resistance genes such as bla_{TEM}, *strA*, *aadA*, *sul1*, *sul2*, *tetA*, *tetB*, *tetC*, and *tetG* was carried out using three multiplex-PCRs (Fig. 1). All the 14 (100%) ampicillin-resistant strains harbored bla_{TEM}, *temA*, and *temB* with sizes 859 bp, 867 bp, and 798 bp, respectively. A comparison between sequenced fragments of bla_{TEM}-positive strains and available sequences in the GenBank revealed similar identity (95%) with bla_{TEM}. All 25 streptomycin-resistant strains harbored the *strA* gene, and only 14 (56.0%) harbored the *aadA* gene. Both *strA* and *aadA* genes were present in 11 (44.0%) strains. Ten of the 27 (37.0%) sulfonamide-resistant strains harbored *sul1*, and 24 (88.8%) harbored *sul2*, whereas 7 (25.9%) strains had both *sul1* and *sul2*. Of the 33 tetracycline-resistant strains, 23 (69.6%) contained the *tetA* gene, 5 (15.1%) had *tetB*, and 4 (12.1%) had *tetC*. No strain was positive for *tetG*. A combination of *tetA*+*tetB* genes was found in one strain (STM DT104 6344/95). One tetracycline-resistant clinical strain (STM 0287/69) did not harbor any of the 4 tetracycline-resistant genes.

Detection of Integrons

Class 1 integrons were present in 33 (70.2%) of the strains: 13 clinical and 20 zoonotic. Three strains carried one integron, 19 strains carried 2, and 11 strains carried 3. All except one integron-positive strain carried the 210-bp size integron. Sequencing analysis showed that the 210-bp fragment is a part of the *pur*G gene that encodes the enzyme phosphoribosylformylglycinamidine synthetase (GenBank Accession No. AF151984). In the other profiles, 5 additional amplicons were detected (650 bp, 800 bp, 1,000 bp, 1,200 bp, 1,900 bp). Integron 650 bp contained the *sat* gene that encodes the enzyme streptothricin acetyltransferase and confers resistance to streptothricin. Intergron 800 bp harbored *dfrA*7 that encodes the enzyme dihydrofolate reductase

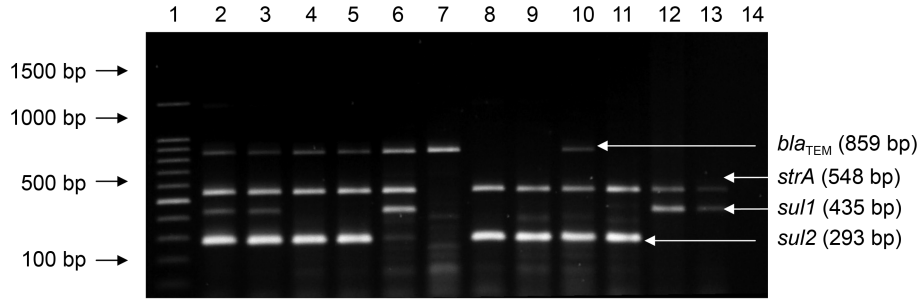

Fig. 1. Representative gel of multiplex-PCR for detection of resistance genes.
DNA bands are indicated by bla_{TEM} (859 bp), *strA* (548 bp), *sul1* (435 bp), and *sul2* (293 bp). Lanes 1–14: DNA size standard (100-bp ladder), positive control, 2625/05, 1204/05, 110187/70, 3866/05, 3865/05, 3077/05, 5229/05, 2559/05, 32/05, 447/05, 447/05, negative control.

Table 3. Phenotypic and genotypic characteristics of zoonotic and human *S.* Typhimurium strains.

Strain No.	Source	PT[a]	Resistotypes (profiles)	Resistance genes present	Plasmid sizes (kb)	Integrons (kb)	PFGE profiles
5553/04	Animal[c]	nd	$	N	90	N	X26
2593/04	Cattle	nd	$	N	90	N	X16
1377/05	Frog	nd	$	N	2.0, 28.9, 95	N	X39
1621/05	Chicken	nd	$	N	90	N	X8
2560/05	Chicken	nd	$	N	3.8, 2.6	N	X30
2552/05	Cattle	nd	$	N	13, 34.3, 65.2	N	X37
3000/05	Fish	nd	$	N	13, 34.3, 65.2	N	X21
2553/05	Cattle	104[b]	$	N	N	N	X22
0504/69	Human	104	$	N	2.7	N	X7
3215/03	Cattle	104	CE (P1)	N	N	N	X15
1234/04	Animal	nd	Su (P2)	*sul2*	N	0.21, 0.6	X26
402/05	Chicken	nd	T (P3)	*tetA*	90	0.21, 0.8	X17
2599/05	Animal	104	T (P3)	*tetA*	N	N	X25
28723/77	Human	nd	T (P3)	*tetB*	N	0.21	X6
3865/05	Chicken	104	A, Nx (P4)	bla_{TEM}	N	N	X30
01/06	Human	104	CE, CL (P5)	N	N	0.21, 0.8	X19
7456/04	Cattle	104	Su, T (P6)	*sul2, tetA*	N	0.21, 0.6	X9
5067/05	Animal	nd	Nx, T (P7)	*tetA*	N	0.21, 0.6	X27
3503/05	Chicken	nd	Nx, T (P7)	*tetA*	N	N	X11
2555/05	Cattle	104	Nx, T (P7)	*tetA*	N	N	X10
8322/03	Chicken	nd	S, Su, T (P8)	*strA, sul2, tetA*	11.3, 22.8, 50	0.21, 1.0	X28
6324/03	Chicken	nd	S, Su, T (P8)	*strA, sul2, tetA*	11.3, 22.8, 50, 90	0.21, 1.0	X28
4524/05	Chicken	nd	S, Su, T (P8)	*strA, sul2, tetA*	3.1, 11.3, 22.8, 50	0.21, 1.0	X31
0287/69	Human	104	S, Su, T (P8)	*strA, sul1, sul2*	11.3, 22.8, 50	0.21, 1.0	X3
113254/70	Human	104	S, Su, T (P8)	*strA, sul2, tetA*	2.6, 11.3, 22.8	0.21, 1.0	X1
32/05	Human	nd	S, Su, T (P8)	*strA, sul2, tetA*	11.3, 22.8, 50	0.21, 1.0	X18
254/98	Chicken	nd	G, Nx, S, T (P9)	*strA, tetB*	5.4	0.21, 1.0	X12
981/04	Animal	nd	Nx, S, Su,T (P10)	*strA, sul2, tetA*	22.8, 50, 90	0.21, 1.0	X36
5231/05	Cattle	104	S, Su, T, CE (P11)	*strA, sul2, tetA*	N	0.21, 0.6	X21
3068/98	Chicken	104	A, G, N, S, T (P12)	bla_{TEM}, *strA, temB, tetB, aadA*	2.0, 2.2	0.21, 1.0	X32
5229/05	Chicken	nd	K, S, Su, T, Tm (P13)	*strA, sul2, tetA*	11.3, 22.8, 50, 90	0.21, 0.6, 1.0	X27
447/05	Human	104	K, S, Su, Sxt, T, Tm (P14)	*strA, sul1, tetA, aadA*	3.4, 4.3, 90	0.21, 1.9	X24

Table 3. Continued.

Strain No.	Source	PT[a]	Resistotypes (profiles)	Resistance genes present	Plasmid sizes (kb)	Integrons (kb)	PFGE profiles
87098/70	Human	104	CE, CL, KF, S, Su, T (P15)	*strA, sul2, tetA , aadA*	11.3, 22.8, 50, 90	0.21, 0.6, 1.0	X1
6344/95	Animal	104	C, CE, AMC, KF, S, Su, T (P16)	*strA, sul1, sul2, tetA, tetB, aadA*	11.3, 22.8, 50, 90	0.21, 1.0, 1.2	X14
5532/04	Animal	nd	A, N, Su, KF, CE, CL, AMC (P17)	bla_{TEM}, *sul2*	2.0, 2.2	0.21, 0.6	X35
3866/05	Chicken	nd	A, Nx, S, Su, Sxt, T, Tm (P18)	bla_{TEM}, *strA, sul1 tetA, temB, aadA, temA*	2.2, 3.4	1.2, 1.9	X38
3077/05	Swine	nd	K, S, Su, T, Tm, AMC, KF, CE (P19)	*strA, sul2, tetA*	10.6, 22.8	0.21, 0.6, 1.0	X34
95893/70	Human	104	A, C, S, Su, T, K, KF, CL (P20)	bla_{TEM}, *strA, sul1, sul2, temB, aadA, temA, tetC*	2.6, 3.0, 7.5, 11.3, 32.1, 90	0.21, 1.0, 1.2	X5
79495/70	Human	104	A, C, S, Su, T, AMC, K, KF (P21)	bla_{TEM}, *sul1, sul2, temB, aadA, temA, tetC*	34.3, 90	0.21, 1.0, 1.2	X1
30822/70	Human	104	A, C, K, Su, T, AMC, KF, CL (P22)	bla_{TEM}, *sul1, sul2, temB, aadA, temA, tetC*	32.3, 90	0.21, 1.0	X2
3079/05	Cattle	nd	A C, S, Su, T, K, Nx, Sxt, Tm, KF (P23)	bla_{TEM}, *strA, sul1, sul2, tetA, temB, aadA, temA*	11.3, 22.8, 50, 90	0.21, 0.6, 1.9	X29
2559/05	Cattle	nd	A, C, S, Su, T, Nx, Sxt, Tm, G, KF (P24)	bla_{TEM}, *strA, sul2, temB, aadA, tetB*	4.9, 83.4, 90	0.21	X13
2625/05	Chicken	nd	A, C, S, Su, T, K, Nx, Sxt, Tm, AMC, KF (P25)	bla_{TEM}, *strA, sul1, sul2, tetA, temB, aadA, temA*	10.6, 28.3, 52.6	0.21, 0.6, 1.9	X20
110187/70	Human	104	A, C, S, Su, T, K, Tm, AMC, KF, CE, CL (P26)	bla_{TEM}, *strA, sul2, temB, aadA, temA*	34.3, 90	0.21, 0.6, 1.0	X4
02/06	Human	nd	A, C, S, Su, T, Cip, G, K, Nx, Sxt, Tm (P27)	bla_{TEM}, *strA*, *sul1*, *tetA*, *temB*, *aadA*, *temA*	2.6, 3.0, 7.5, 11.3, 22.8, 50	0.21, 0.6, 1.9	X23
1204/05	Chicken	104	A, C, S, Su, T, G, K, KF, AMC, CRO, CTX, CE, CL (P28)	bla_{TEM}, *strA, sul2, tetA, temA*	3.4, 90	0.21	X33
196/05	Human	nd	A, S, Su, T, XNL, KF, CL, CE, CXM, CRO, CTX, CAZ, AMC, FEP, ATM (P29)	bla_{TEM}, *strA, sul2, tetA, temB*	3.4, 4.3, 11.3, 22.8, 50	0.21, 1.0, 1.2	X18

$, Sensitive; Nx, nalidixic acid; A, ampicillin; S, streptomycin; Cip, ciprofloxacin; Sxt, trimethoprim/sulfamethoxazole; T, tetracycline; C, chloramphenicol; Tm, trimethoprim; G, gentamicin; K, kanamycin; Su, sulfonamides; XNL, ceftiofur; FEP, cefepime; ATM, aztreonam; KF, cephalothin; CAZ, ceftazidime; CE, cephradine; CRO, ceftriaxone; CL, cephalexin; CXM, cefuroxime; CTX, cefotaxime; AMC, amoxicillin–clavulanic acid.

[a]PT, Phage Type; [b]Phage type DT104 determined by PCR; [c]Unknown animal hosts; nd, not determined; N, no integron/gene/plasmid.

and confers resistance to trimethoprim. Integron 1,000 bp harbored the *aadA2* gene that encodes for aminoglycoside adenyltransferase AAD(3") and confers resistance to streptomycin–spectinomycin. Integron 1,200 bp harbored the *pse-1* gene that encodes for beta-lactamase and confers resistance to ampicillin. Integron 1,900 bp harbored the *dfrA12* gene located near the 5' end that encodes for dihydrofolate reductase and confers resistance to trimethoprim, and the *aadA2* gene located near the 3' end that encodes aminoglycoside adenyltransferase AAD(3") and confers resistance to streptomycin–spectinomycin.

Plasmid Analysis

Thirty-five (74.4%) strains (12 human, 23 animal) harbored 1–6 plasmids (2.0 to 95 kb) each (Table 3). Two human strains (STM 02/06 and STM 95893/70) had 6 plasmids (Table 3). The predominant plasmid was 90 kb, present in 17 (48.5%) strains, followed by 22.8 kb (n=14), 11.3 kb (n=13), and 50 kb (n=12).

Genotyping

PFGE of *Xba*I-digested chromosomal DNA subtyped all the 47 strains into 39 pulsotypes (PFPs) with DNA fragments

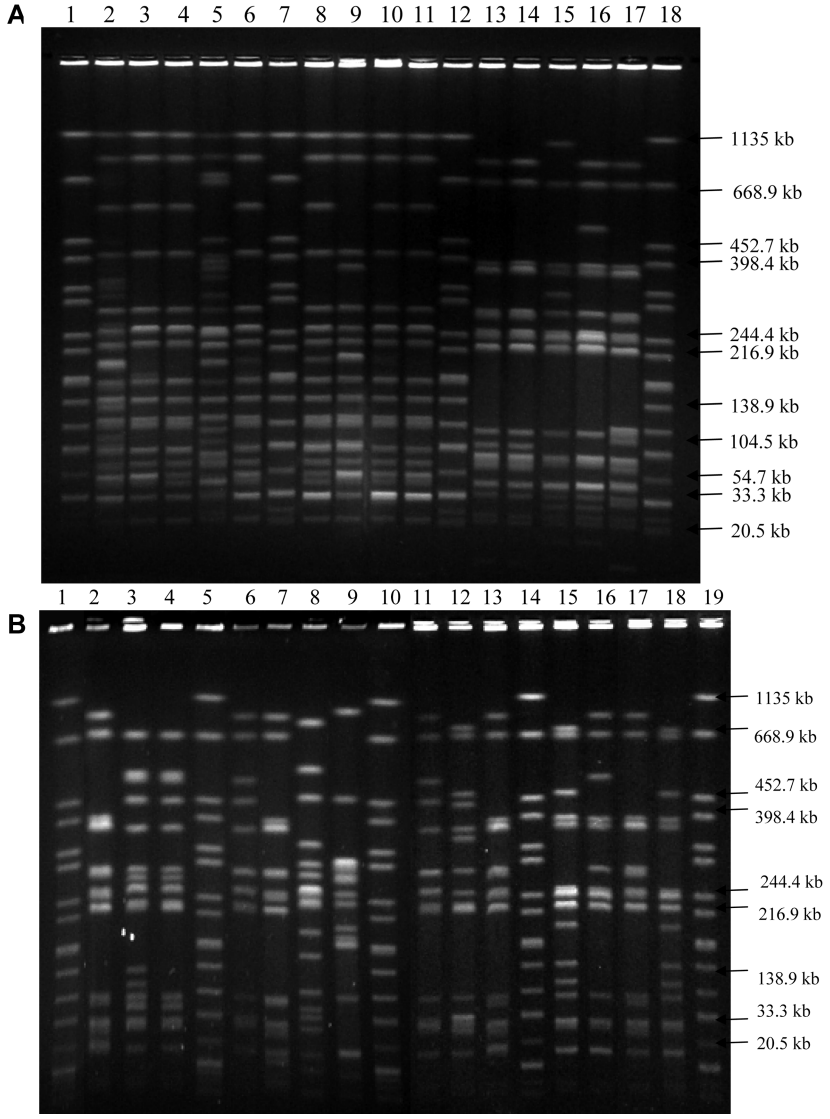

Fig. 2. PFGE profiles of selected human and zoonotic *S*. Typhimurium strains.
A. Representative *Xba*I-PFGE profiles of selected human *S.* Typhimurium strains. Lanes 1, 7, 12, 18: *Salmonella* H9812 marker strain; lanes 2–6, 8–11, 13–17: 95893/70, 110187/70, 13254/70, 0504/69, 0287/69, 30822/70, 28723/77, 87098/70, 79495/70, 32/05, 196/0, 447/05, 01/06, 02/06. **B**. Representative *Xba*I-PFGE profiles of selected zoonotic *S.* Typhimurium strains. Lanes 1, 5, 10, 14, 19: *Salmonella* H9812 DNA marker strain; lanes 2–4, 6–9, 11–13, 15–18: 6321/03, 981/04, 5532/04,1234/04, 3079/05, 3503/05, 3866/05, 5553/04, 2593/04, 5231/05, 2560/05, 2553/05, 3000/05, 3865/05.

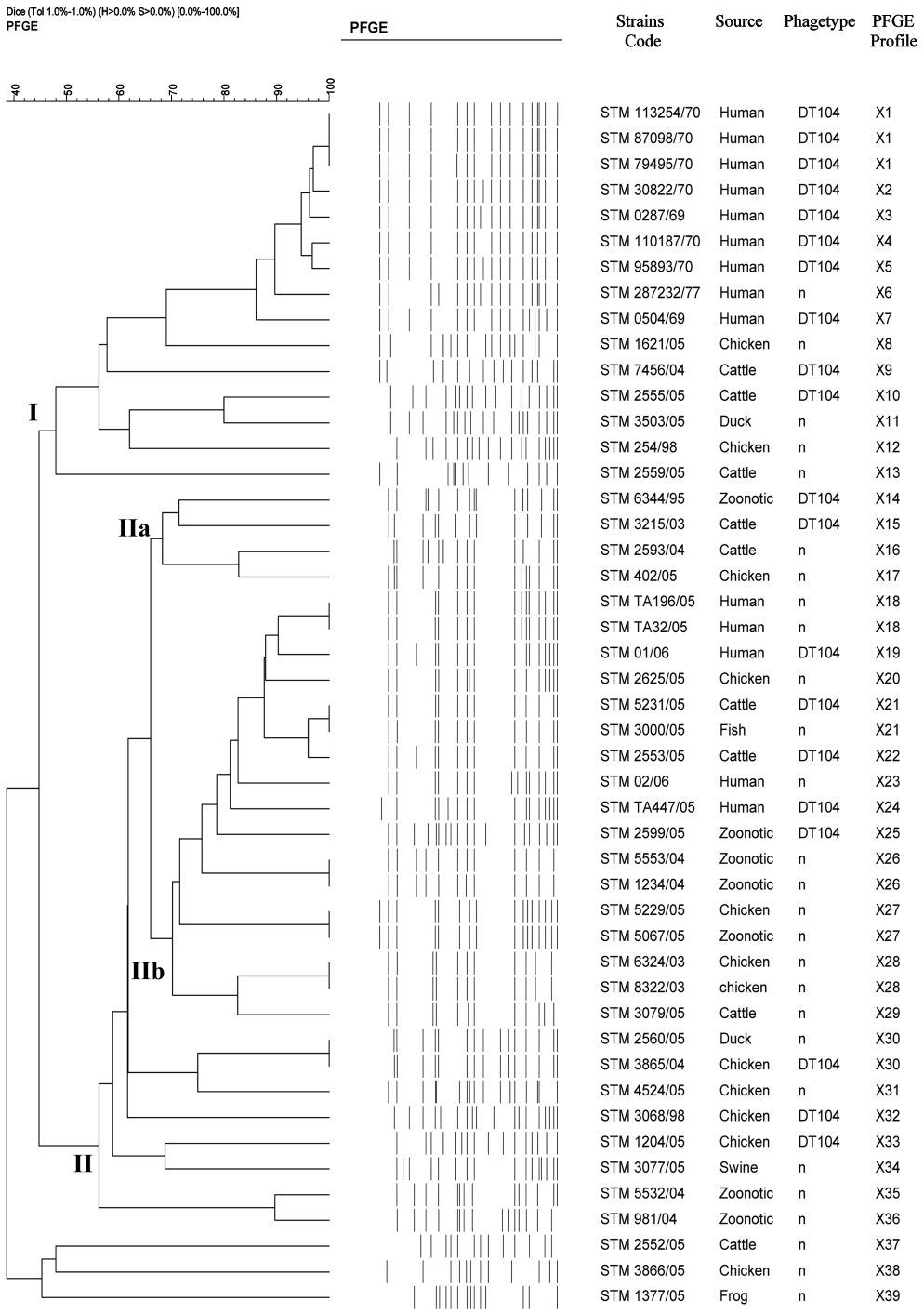

Fig. 3. Dendrogram of cluster analysis of *S.* Typhimurium strains generated by Gel Compar software using the unweighted pair group arithmetic means (UPGMA) methods. n=not determined.

ranging from 11 to 19 with sizes from 20.5 kb to 1,135 kb. Wide genetic diversity was found among the strains (F= 0.4 to 1.0). Among these 39 different pulsotypes, 6 were represented by 2 strains each. Three human strains of phage type DT104 isolated in 1970 were indistinguishable (Profile X1). The rest of the PFGE profiles were unique. Among the 14 human strains, there were 11 PFPs (Fig. 2A), whereas the 33 zoonotic strains had 28 PFPs (Fig. 2B).

Cluster analysis of the pulsotypes of the 47 *S.* Typhimurium strains based on 70% similarity generated 2 major clusters,

I and II (Fig. 3). Cluster I consisted of 10 strains (8 MDR, 1 drug-sensitive human strain, and 1 drug-sensitive zoonotic strain). Cluster II consisted of 21 strains, comprising 16 PFPs divided into 2 subclusters, IIa and IIb. Subcluster IIa consisted of 4 animal strains isolated between 1995 and 2005, each having a unique PFP, 3 MDR, and 1 drug-sensitive strains. Subcluster IIb consisted of 17 strains, comprising 12 PFPs. The rest of the 15 PFPs were not included in any distinctive cluster, and each unique profile was represented by 1 strain, except PFP X30, which was represented by 2 strains. The majority of "older" human strains isolated from 1969 to 1977 were grouped in cluster I with similarity >85%, which suggests that they were very closely related and were distinctly different from those isolated in recent years.

DISCUSSION

In Malaysia, there is little information on the prevalence of *Salmonella* Typhimurium. Although the number of *Salmonella* infections caused by *S*. Typhimurium remains relatively low, it is still important because of increasing isolation of this particular serovar from food and animals in Malaysia (unpublished data).

Phage typing is important for the epidemiology of *Salmonella* and has been used to describe pandemic clones of *S.* Typhimurium DT104. In Malaysia, there are no phage typing facilities for *S.* Typhimurium, and therefore this is the first report from this region on the differentiation of *S*. Typhimurium by PFGE and PCR. The use of PCR to serotype *S*. Typhimurium has been successfully reported by others [4].

A high level of multidrug resistance was found among the 47 zoonotic and human strains of *S*. Typhimurium, 57.4% being resistant to 3 or more groups of antimicrobial agents. The most common pattern shared among the strains was ACSSuT. In this study, only 4 *S*. Typhimurium DT104 had this pattern.

High rates of resistance to tetracycline (70.2%) were found among the strains. This finding was lower than those from Korea where 90.0% of the *S*. Typhimurium animal strains were resistant to tetracycline [33], probably due to this agent being widely used in animal feed as a growth enhancer [9]. High levels of resistance to streptomycin (53%) and ampicillin (29.7%) were also found, but these were lower than those from Taiwan, where 82% and 76% of 242 strains were resistant to streptomycin and ampicillin, respectively [19].

We found 57% of the strains were resistant to sulfonamides, being similar to that reported by Lailler *et al*. [18], where 56% of 168 *S.* Typhimurium strains from cattle, pigs, and poultry were resistant to sulfonamides. In addition, we also observed 27.6% resistance to nalidixic acid and 19% to trimethoprim. Numerous studies have reported the increase in resistance of *S.* Typhimurium strains to nalidixic acid and trimethoprim [6, 17], probably as a result of the use of these agents in the treatment of invasive gastrointestinal infections [1] and in animal feeds [30]. Besides the high resistance to nalidixic acid, resistance to ciprofloxacin was found in one human strain isolated in 2006.

Resistance rates of *S*. Typhimurium strains to cephalosporins were higher in the human than in the zoonotic strains, probably due to the use of cephalosporin antibiotics in the treatment of salmonellosis [10].

A multiplex-PCR was applied for accurate detection of antibiotics resistance genes. Our results show that the predominant tetracycline-resistant gene *tetA* was present in both human and zoonotic strains, followed by *tetB* and *tetC* genes. None of our tetracycline-resistant strains harbored the *tetG* gene. However, Ng *et al*. [22] found that among 31 *S.* Typhimurium strains isolated in Canada, *tetG* was the predominant tetracycline resistance gene present, followed by *tetB*.

Sulfonamide-resistant gene *sul2* was detected among our 27 sulfonamide-resistant strains. The *sul2* gene has been found associated with plasmids and not with the class 1 integron [5], but in our study the *sul2* gene was found alone in 17 integron-positive strains. The *sul1* gene was detected as part of the class 1 integron in 10 Malaysian strains. Seven strains harbored both *sul1* and *sul2* genes, indicating that these strains probably carried class 1 integrons and plasmids.

The *aadA* gene, which confers resistance to streptomycin–spectinomycin, was found as part of the class 1 integron, in agreement with previous studies that it is present in the variable region of class 1 integrons in *S*. Typhimurium [6, 25].

The *strA* gene was the most frequently found in all streptomycin-resistant strains, and has been reported to be associated with plasmids [25]. Both *strA* and *aadA* genes were present in 11 strains, which most probably carried plasmids containing the *strA* gene and the class 1 integron harboring the *aadA* gene.

Class 1 integrons were present in 33 (70.2%) strains, indicating a wide distribution of this mobile genetic element among the Malaysian *Salmonella* Typhimurium strains. Most of the integron-positive strains carried the 210-bp integron, which appears to be unique for *S*. Typhimurium [12] and is part of the *pur*G gene. The 800-bp integron harbored *dfrA*7. Sequence analysis of the 1,000-bp amplicon of different integron profiles showed the presence of *aadA2* genes. The persistence and spread of these genes in different *Salmonella* serotypes might be associated with the extensive use of streptomycin in food animals. Similar results were reported previously [6, 14]. The 1,200-bp region carried the *pse-1* gene (AF153200). Similar integrons were reported in several previous studies [6, 22]. According to Guerra *et al*. [14], the presence of

integrons with variable regions of 1,000 bp (*aadA2*) and 1,200 bp (*pse-1*) is very common among multiple resistant DT104 strains. The 1,900-bp amplicon was found in multidrug-resistant strains (resistant to more than 5 antimicrobial agents), and sequencing analysis showed that the 1,900-bp amplicon carried 2 genes; the *dfrA12* gene, which conferred resistance to trimethoprim, and the *aadA2* gene, which conferred resistance to streptomycin–spectinomycin. Guerra *et al*. [15] also reported both these genes (*aadA2* and *dfrA12*) in the 1,900-bp class 1 integron in *Salmonella enterica* serotype [4,5,12:i:-] isolated in Spain. Whether the gene cassettes in the integrons are associated with *Salmonella* Genomic Island I as commonly reported [8] will be determined in future work.

Salmonellae can harbor plasmids of different molecular sizes, ranging from 1 to 200 kb [27]. In this study, 22 different plasmid profiles with plasmids ranging from 2.0 to 95 kb were obtained. Ten small plasmids, 2.0, 2.2, 2.64, 3.1, 3.4, 3.8, 4.3, 5.4, 10.6, and 11.3 kb, were identified. Similar small plasmid sizes were reported by Biendo *et al*. [7] in France and Foley *et al*. [13] in the U.S.A.

Seventeen (48.5%) plasmid-positive strains harbored the 90-kb plasmid, whereas one strain had the 95-kb plasmid. This particular sized plasmid has been called the *Salmonella* serovar-specific plasmid, and was reported in several studies [3, 21].

No plasmid was detected in 11 drug-resistant *S.* Typhimurium strains in this study. However, the gain or loss of plasmids is well established, and therefore the analysis of the plasmid profile is not a definitive typing method. During plasmid extraction, shearing of large-plasmid DNA and coprecipitation with the chromosomal DNA are usually reported as one of the causes of plasmid loss [16]. According to Olsen *et al*. [24], the absence of plasmid in the resistant strains could be due to plasmid instability, a common phenomenon among salmonellae.

No distinct association was found between the resistant phenotypes and *Xba*I-pulsotypes by cluster analysis of the 47 *S*. Typhimurium strains. PFGE did not distinguish the drug sensitive and resistant strains, as they shared similar pulsotypes. The human strains isolated between 1969 and 1977 were grouped in one cluster. All except one (28723/77) were DT104, indicating that this phage type was prominent in the *S.* Typhimurium population at that period (1969–1977). These strains were very similar (85%), and were closely related. We identified 22 unique PFGE patterns among the zoonotic *S*. Typhimurium over a period of 10 years (1995–2005). The high diversity of PFGE subtypes suggests that the infections in different animal hosts were from various sources. The majority of older human strains isolated from 1969 to 1977 were grouped in cluster I with similarity >85%, which suggests that they were very closely related and were distinctly different from those isolated in recent years.

In conclusion, high rates of resistance were found among the strains indicated by the prevalence of resistance genes. Approximately 70.2% and 74.4% of the strains carried class 1 integrons and plasmids, which may explain the dissemination of antimicrobial resistance genes through these elements. The existence of multidrug-resistant *S.* Typhimurium to cephalosporins was also observed. PFGE analysis showed that the strains were very diverse and no genotype seemed to predominate and persist over the years.

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