Characterization of *Salmonella enterica* Serovar Typhimurium and Monophasic *Salmonella* Serovar 1,4,[5],12:i:- Isolates in Thailand

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Duplex PCR was developed to screen *Salmonella enterica* serovar Typhimurium phage type DT104 and related strains in Thailand because a phage typing laboratory of serovar Typhimurium is not available. Of 46 isolates of serovar Typhimurium and 32 isolates of *S. enterica* serovar 1,4,[5],12:i:-, 15 (33%) and 30 (94%) were duplex PCR positive, respectively. All isolates were submitted for phage typing to analyze the specificity of the PCR assay. Among serovar Typhimurium isolates that yielded positive duplex PCRs, only seven isolates were phage types DT104 or U302, and eight isolates were undefined types, whereas the negative PCR isolates were either other phage types, including DT7, DT12, DT66, DT79, DT166, DT170, DT193, and DT208 or an undefined type. The serovar Typhimurium and serovar 1,4,[5],12:i:- isolates that were duplex PCR positive were further subtyped by using XbaI PFGE to reveal their genetic relatedness. All serovar Typhimurium phage type DT104 strains had indistinguishable chromosomal patterns. The isolates of phage type U302 and most of the serovar 1,4,[5],12:i:- isolates that were duplex PCR positive yielded similar pulsed-field gel electrophoresis patterns. The patterns of PCR-negative isolates distinctly differed from the patterns of PCR-positive isolates. A total of 26% of all isolates had a dominant R-type ACSSuTG that was not found in the isolates of phage type DT104.

Salmonella enterica serovar Typhimurium phage types DT104 and U302 are considered zoonotic and life-threatening pathogens (1, 15). These strains are commonly associated with multiple-drug-resistant (MDR) characteristics that are carried by genetic elements in chromosome (4, 6, 13, 18). Serovar Typhimurium is one of the common serovars found globally, including Thailand. However, serovar Typhimurium has not caused a critical outbreak in Thailand, and the incidence of these strains has not increased as in some countries (1, 3, 13, 15).

During the last decade *S. enterica* serovar 1,4,[5],12:i:- was one of the five most common serovars found in Thailand (3). This serovar could be a monophasic variant of serovar Typhimurium or other serovars. Interestingly, the strain within this serovar was reported to be a related strain to DT104 and had a phage pattern similar to that of U302 (8).

In the present study, the duplex PCR was developed to investigate phage type DT104 and its related strains among the isolates of the serovars Typhimurium and 1,4,[5],12:i:-. The PCR was expected to be used as a screening assay in regions where a phage type laboratory is not available. The PCR was evaluated by using phage typing and pulsed-field gel electrophoresis (PFGE). Consequently, the subtype information and antimicrobial profiles of these serovars could reveal characteristics of the strains of serovar Typhimurium and their monophasic serovars in Thailand, where the distribution of *Salmonella* serovars differs from that seen in other countries.

MATERIALS AND METHODS

A total of 46 isolates of serovar Typhimurium and 32 isolates of *Salmonella* 1,4,[5],12:i:- were collected between 2000 and 2001 from different geographic areas in Thailand and were serotyped according to the Kauffman-White scheme (12) at the World Health Organization (WHO) National *Salmonella* and *Shigella* Centre, Bangkok, Thailand. The origins of the isolates were from human, frozen meat, food, and feed. The antimicrobial susceptibility test was performed by the disk diffusion method as described by the National Committee for Clinical Laboratory Standards (NCCLS) (16). The disks included 10 µg of ampicillin, 30 µg of chloramphenicol, 10 µg of gentamicin, 30 µg of kanamycin, 10 µg of streptomycin, 1.25/23.75 µg of sulfatrimetoprim, and 30 µg of tetracycline.

The suspension of isolates were heated at 100°C for 10 min and used as DNA templates. The pair of DT104 primers, including DT104-F (5'-GTCAGCAGT GTATGGAGCGA-3') and DT104-R (5'-AGTAGCGCCAGGACTCGTTA-

FIG. 1. Duplex PCR of *Salmonella* isolates. The 162-bp fragment is specific to phage type DT104. The 261-bp fragment is specific to serovar Typhimurium. Lane M, molecular marker pUC18 digested with HaeIII; lane 1, serovar Typhimurium phage type DT104; lane 2, serovar Typhimurium phage type DT12; lanes 3, 6, and 8, serovar 1,4,[5],12:i:-; lane 4, serovar Typhimurium RDNC; lane 5, *Salmonella* serovar Mbandaka.

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TABLE 1. Results of duplex PCR and phage types

Molecular size (bp) ^a	Phage types and/or serovars	No. of isolates
261, 162	DT104	4
261, 162	U302	3
261, 162	1,4,[5],12:i:-	30
261, 162	RDNC ^b	8
261, -	1,4,[5],12:i:-	2
261, -	RDNC	13
261, -	DT7, D12, D66, D79, D166, D170, D193, and D208	18

^a The molecular weights of PCR products are given in base pairs as determined by PCR.

^b RDNC, reaction does not conform to Typhimurium phage patterns.

3'), was derived from a specific sequence of 16S-to-23S spacer region of phage type DT104 and its related phage type, U302 (17). The other primer pair including MDH-F (5'-TGCCAACGGAAGTTGAAGTG-3') and MDH-R (5'-CGCATTCACACGCCCTTC-3'), encompassed *mdh* (malic acid dehydrogenase gene) of serovar Typhimurium (GenBank accession number X61029) (14). The PCR amplification was performed in 10 µl of a solution containing 1.5 mM MgCl₂, 50 µM concentrations of each deoxynucleoside triphosphate, 0.4 µM concentrations of each primer, and 0.5 U of *Taq* polymerase (Qiagen). The PCR conditions were an initial incubation at 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, with a final extension at 72°C for 5 min by using GeneAmp Model 9600 thermocycler (Applied Biosystems). A number of PCR products were verified by using an ABI BigDye terminator sequencing kit (Applied Biosystems).

The strains used for PCR positive control were four isolates of serovar Typhimurium DT104 from the Microbiological Diagnostic Unit, University of Melbourne, Melbourne, Australia. Twelve negative control isolates were *S. enterica*

TABLE 2. Origins, R types, phage types, and PFGE patterns of duplex PCR-positive isolates

Isolate no.	Specimen type	Origin	R type ^a	PT/ ^b	PFGE pattern
H1	Urine	Songkla	ACSSuTG	i:	E
H2	Blood	Songkla	ACSSuTG	RDNC	U7 (+80, 50)
H3	Rectal swab	Songkla	ACSSuTG	i:	U8 (+80, 70)
H4	Rectal swab	Samut Songkram	ACSSuTG	i:	U5 (+150)
H5	Stool	Tak	ACSSuTG	i:	U4 (+130)
H6	Rectal swab	Bangkok	ACSSuTG	i:	U6 (+170)
H7	Stool	Ratchaburi	ACSSuTG	i:	U4 (+130)
H8	Stool	Buriram	ACSSuTG	RDNC	U7 (+80, 50)
H9	Rectal swab	Trang	ACSSuTG	U302	U6 (+170)
H10	Stool	Songkla	ACSSuTG	RDNC	U3 (+80)
H11	Pus	Ratchaburi	ACSSuTG	i:	V
H12	Stool	Songkla	ACSSuTG	i:	U2 (+70)
H13	Food	Bangkok	ACSSuTG	RDNC	U1 (+50)
H14	Rectal swab	Bangkok	ACSSuTG	i:	U7 (+80, 50)
H15	Blood	Bangkok	ACSSuTG	i:(U302) ^c	U5 (+150)
H16	Stool	Songkla	ACSSuTG	i:	U
H17	Rectal swab	Songkla	ACSSuTG	i:	U2 (+70)
H18	Stool	Bangkok	ACSSuTG	i:(U302)	X
H19	Stool	Bangkok	ACSSuTG	i:(U302)	U4 (+130)
H20	Stool	Trang	ACSSuTK	i:	U4 (+130)
H21	Stool	Songkla	ACSSuT	i:	U
H22	Rectal swab	Bangkok	ACSSuT	U302	U
H23	Stool	Bangkok	ACSSuT	RDNC	W
H24	Stool	Nonthaburi	ACSSuT	i:	U
H25	Stool	Chiangrai	ASSuTG	i:	U1 (+50)
H26	Rectal swab	Bangkok	ASSuTG	i:	U3 (+80)
H27	Urine	Nonthaburi	ASSuTK	i:(U302)	U11 (+70, 80, 310)
H28	Rectal swab	Bangkok	ACSTG	i:	U
H29	Rectal swab	Trang	ASSuT	i:	U9 (+80, 170)
H30	Pus	Bangkok	ASSuT	i:	U
H31	Stool	Trang	ASSuT	i:	U7 (+80, 50)
O32	Chicken meat	Bangkok	ASSuT	U302	U3 (+80)
H33	Rectal swab	Bangkok	ACST	RDNC	U
O34	Food	Bangkok	ACST	DT104	D
O35	Food	Bangkok	ACST	DT104	D
H36	Stool	Suratthanee	ASSu	i:	U3 (+80)
H37	Rectal swab	Bangkok	ASuT	i:	U10 (+30, 50, 130)
H38	Blood	Bangkok	CST	i:	U1 (+50)
H39	Stool	Ratchaburi	CST	RDNC	U3 (+80)
H40	Blood	Bangkok	CST	RDNC	D1 (+300)
H41	Blood	Songkla	AT	i:	U3 (+80)
H42	Stool	Bangkok	SuT	i:	Y
H43	Stool	Ratchaburi	SSu	i:	U5 (+150)
O44	Chicken meat	Bangkok	A	DT104	D
O45	Chicken meat	Bangkok	S	DT104	D

^a Antimicrobial agents: A, 10 µg of ampicillin; C, 30 µg of chloramphenicol; S, 10 µg of streptomycin; Su, 1.25/23.75 µg of sulfatrimetroprim; T, 30 µg of tetracycline; G, 10 µg of gentamicin; K, 30 µg of kanamycin.

^b RDNC, reaction does not conform to Typhimurium phage patterns. PT, phage types; i, *S. enterica* subspecies 1 serovars 1, 4, [5], and 12:i:-.

^c i (U302); 1,4,[5],12:i:- that showed the same phage pattern as U302.

TABLE 3. Origins, R types, and phage types of the duplex PCR-negative isolates

Isolate no. ^a	Specimen type	Origin	R type ^b	PT/ ^c
H46	Stool	Bangkok	ACSSuT	i
O47	Chicken meat	Bangkok	ASSuT	DT193
O48	Chicken meat	Bangkok	ASSuT	DT193
O49	Chicken meat	Bangkok	ASSuT	DT12
O50	Chicken meat	Bangkok	ASSuT	DT193
O51	Frozen sea food	Nonthaburi	AST	DT7
O52	Frozen raw material	Chiangmai	ASuT	DT170
O53	Food	Bangkok	CST	RDNC
O54	Food	Bangkok	CST	RDNC
H55	Rectal swab	Songkla	SSuT	DT208
O56	Chicken meat	Bangkok	SSuT	RDNC
O57	Chicken meat	Bangkok	SSuT	DT208
O58	Chicken meat	Bangkok	SSuT	DT208
O59	Chicken meat	Bangkok	SK	DT166
O60	Food	N Si Thammarat	SuT	DT208
O61	Frozen chicken	Bangkok	S	RDNC
O62	Chicken meat	Bangkok	S	RDNC
O63	Chicken meat	Bangkok	S	RDNC
O64	Chicken meat	Bangkok	S	RDNC
O65	Chicken meat	Bangkok	S	DT193
O66	Chicken meat	Bangkok	S	RDNC
O67	Frozen raw material	Nonthaburi	Sensitive	RDNC
H68	Stool	Buriram	Sensitive	DT66
H69	Stool	Suratthani	Sensitive	RDNC
O70	Chicken meat	Bangkok	Sensitive	RDNC
O71	Chicken meat	Bangkok	Sensitive	DT79
O72	Chicken meat	Bangkok	Sensitive	DT79
O73	Chicken meat	Bangkok	Sensitive	RDNC
O74	Chicken meat	Bangkok	Sensitive	DT12
O75	Chicken meat	Bangkok	Sensitive	RDNC
O76	Chicken meat	Bangkok	Sensitive	i
O77	Chicken meat	Bangkok	Sensitive	DT7
O78	Chicken meat	Bangkok	Sensitive	DT79

^a Isolates 59, 67, 68, and 69 were analyzed by PFGE as negative control strains.

^b Antimicrobial agents: A, 10 µg of ampicillin; C, 30 µg of chloramphenicol; S, 10 µg of streptomycin; Su, 1.25/23.75 µg of sulfatrimetoprim; T, 30 µg of tetracycline; G, 10 µg of gentamicin; K, 30 µg of kanamycin.

^c RDNC, reaction does not conform to Typhimurium phage patterns. PT, phage types; i, *S. enterica* subspecies I serovar 1,4,[5],12:i:-.

serovars Anatum, Choleraesuis, Derby, Enteritidis, Mbandaka, Mgulan, Paratyphi, Rissen, Stanley, Swazengurd, and Weltevreden and *Escherichia coli*.

Phage typing was performed at Microbiological Diagnostic Unit, University of Melbourne, and at the WHO Danish Veterinary Laboratory, Copenhagen, Denmark. Phage types were determined based on the method of Anderson et al. (2), according to serovar Typhimurium phage typing scheme of the Public Health Laboratory Service, Colindale, United Kingdom.

All of the isolates that gave double bands in PCR and four isolates (number O59, O67, H68, and H69) that gave only one band in PCR, were further subtyped by using XbaI PFGE at 14°C, 6 V/cm, and 120° included angle. The pulsed conditions were 5 to 15 s for 7 h, followed by 15 to 60 s for 19 h (11).

RESULTS

PCR and phage types. The duplex PCR product comprised a 261-bp fragment of the *mdh* sequence that was specific for serovar Typhimurium and a 162-bp fragment of the specific sequence in DT104 or U302 (Fig. 1). All of the tested isolates presented the specific fragments to serovar Typhimurium. The other *Salmonella* serovars did not yield this fragment. Of the total isolates tested, 45 isolates yielded two amplification fragments of duplex PCR, and 33 isolates yielded only the band of 261-bp PCR products. Of the 45 duplex PCR positive isolates, 30 isolates were serovar 1,4,[5],12:i:-, 8 isolates were undefined

type (RDNC [for reaction does not conform]), 4 isolates were DT104, and 3 isolates were U302 (Tables 1 and 2).

Among the 33 negative duplex PCR isolates that yielded only the fragment specific to serovar Typhimurium, 2 were serovar 1,4,[5],12:i:-, 13 were RDNC, and 18 were phage types DT7, DT12, DT66, DT79, DT166, DT170, DT193, or DT208 (Tables 1 and 3).

PFGE. The PFGE patterns of the PCR-positive isolates were distinctively different from the patterns of PCR-negative isolates. The patterns were interpreted according to the criteria of Tenover et al. (20). Serovar Typhimurium DT104 isolates O34, O35, O44, and O45 had PFGE patterns that were indistinguishable from one other, and they were reported as pattern D. The isolate number H40 (RDNC) had only one fragment different from pattern D and was designated pattern D1. Pattern E was named for isolate H1 (serovar 1,4,[5],12:i:-), which had more than three fragments different from pattern D (Table 2).

Of three serovar Typhimurium phage type U302 strains, the PFGE pattern of isolate H22 was designated pattern U, and isolates H9 and O32 had one more fragment than pattern U; these were designated patterns U6 (U + 130 bp) and U3 (U + 80 bp), respectively. The isolates that had one to three different fragments from pattern U were described as patterns U1 to U11. The isolates that had PFGE patterns U1 to U11 were either phage type U302 or undefined phage types (RDNC and serovar 1,4,5,12:i:-). The isolates that showed more than three different fragments from pattern U were described as patterns V, W, X, and Y (Table 2). Isolates O59, O57, H68, and H69 were duplex PCR negative, and the isolates of other *Salmonella* serovars had patterns different from either pattern D or U (Fig. 2).

R type. The most common MDR pattern found in the present study was R-type ACSSuTG (24%). All of the isolates

FIG. 2. PFGE XbaI digestion patterns of *Salmonella* Typhimurium phage types DT104 and U302 and *Salmonella* serovar 1,4,[5],12:i:-. Lane M, lambda ladder molecular marker; lane λ, 23 kb of λHindIII; lane 2, pattern U; lanes 1, 3, 6, and 8, patterns U6, U7, U5, and U10, respectively; lanes 4 and 5, patterns of serovar Typhimurium that yielded only one band of duplex PCR; lane 7, PFGE pattern of *Salmonella* serovar Mgulan.

that had this R type were positive in the duplex PCR (Table 2). Most of the isolates that were positive in the duplex PCR and resistant to more than three drug agents belonged to serovar 1,4,[5],12:i:-.

DISCUSSION

Identification and subtyping of MDR serovar Typhimurium is necessary for understanding and control of the associated infection (7, 15). Even though phage typing is a good method for epidemiological surveillance of serovar Typhimurium, a phage typing laboratory is not available in many countries. The duplex PCR was developed for initially screening phage type DT104 and related strains before the isolates were sent to be phage typed abroad. The specificities of two primer pairs were previously described (14, 17) and were also tested in the present study with a variety of *Salmonella* serovars. A number of PCR products were verified by DNA sequencing. Therefore, the PCR assay is reliable as a rapid tool for screening the strains.

This subtyping study of serovar Typhimurium isolates revealed that all of the serovar Typhimurium DT104 strains found were the same clone. The isolates of phage type DT104 did not yield the typical MDR pattern as R-type ACSSuT that has been previously reported in many countries (4, 18). In the present study serovar Typhimurium DT104 was not the dominant phage type. Only four isolates from food and meat specimens were phage type DT104 and had the same PFGE pattern. Only one isolate (H40) from human specimen was possibly related to the DT104 from its PFGE patterns.

Although the number of serovar Typhimurium DT104 and U302 strains in our study was less than expected, the number of MDR serovar 1,4,[5],12:i:- strains that were duplex PCR positive was high. All serovar 1,4,[5],12:i:- strains were isolated from human specimens. There were a number of studies explaining the close clonal relationship of serovars 1,4,[5],12:i:- and Typhimurium. DNA microarray-based typing indicated that four strains of the serovar 1,4,[5],12:i:- showed a close clonal relationship to serovar Typhimurium (10), and IS200 of serovar Typhimurium was also found in the chromosome of serovar 1,4,[5],12:i:- (5). Echeita et al. (9) reported that the sequence-specific 16S-to-23S spacer region of serovar Typhimurium DT104 and U302 appeared in serovar 1,4,[5],12:i:- strains. We also found that 30 isolates (94%) of serovar 1,4,[5],12:i:- yielded the specific fragments of the 16S-to 23S spacer region. It seemed possible that the high proportion of serovar 1,4,[5],12:i:- might result from the undetected of flagella phase by using serology. There was a study using PCR to amplify the gene encoding flagella, and these researchers found that a number of serovar 1,4,[5],12:i:- isolates were positively amplified for this gene (19). We found that all tested isolates of this serovar yielded the band of *mdh* gene that specific to serovar Typhimurium. Therefore, serovar 1,4,[5],12:i:- in our study were very likely to be a monophasic variant of serovar Typhimurium.

The isolates whose phage types were untypeable needed to be further categorized. In the present study, serovar 1,4,5,12:i:- could be either positively or negatively amplified for sequences specific to the 16S-to23S spacer region of DT104. Even though the phage types of serovar 1,4,5,12:i:- could not be determined

because they could not be grouped within specific serovars, it is interesting that 4 of 32 serovar 1,4,5,12:i:- isolates showed the same phage pattern as U302. Moreover, these four isolates had PFGE patterns similar to the pattern of phage type U302. Although most of the serovar 1,4,5,12:i:- isolates did not react with any phage types, many of them had chromosomal patterns that were either the same as or similar to the pattern of serovar Typhimurium U302 (Table 2).

Since the present study focused on the characterization of phage type DT104 or related strains, only 32 isolates of serovar 1,4,5,12:i:- were chosen for subtyping. Therefore, we did not thoroughly evaluate the molecular epidemiology of serovar 1,4,5,12:i:- in Thailand. However, it is very likely that all serovar 1,4,5,12:i:- isolates tested were monophasic variant strains of serovar Typhimurium and that some of them were clones similar to DT104 or U302. In the present study, the antimicrobial-resistant profiles of serovar 1,4,5,12:i:- showed more drug resistance than any subtypes. Greater numbers of the isolates are needed to evaluate their clonal origins and the epidemiology of strains of this monophasic serovar in Thailand. Further studies of their antimicrobial-resistant genes, including determination of the mode of gene acquisition and the relation to DT104 strain and the monophasic variants of other possible *Salmonella* serovars, are required for the epidemiological study of these strains.

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