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Molecular Typing of *Salmonella enterica* serovars Typhimurium, Typhi, and Enteritidis Isolated in Taiwan

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ABSTRACT

Salmonella enterica serovars Typhimurium, Typhi, and Enteritidis are serious food pathogens which may cause human disease and/or animal infections. In an attempt to elucidate the clonal relationship in each of these species, to find the most disseminated and recirculating strains in food poisoning cases, and to discern the possible transmission of these strains from different origins and areas, we have used phage typing, antibiograms and molecular typing methods, such as plasmid profiles, pulsed field gel electrophoresis (PFGE), and random amplified polymorphic DNA (RAPD) to identify subtypes of these *Salmonella* strains. The results showed that in *Salmonella* Typhimurium and Typhi strains, considerable genetic diversity were found while in *S.* Enteritidis, high genetic similarity was observed. Also possibly, the most disseminated and recirculating strains of *S.* Typhimurium and *S.* Enteritidis were identified. Strains of these common subtypes might be the most prevalent strains and transmission of strains between different areas and origins might be possible.

Key words: *Salmonella enterica* serovars, Typhimurium, Typhi, Enteritidis, molecular typing

INTRODUCTION

Pathogenic bacteria, such as *Salmonella* spp., *Staphylococcus aureus*, *Shigella* spp., *Escherichia coli*, *Vibrio parahaemolyticus*, *Yersinia enterocolitica*, *Bacillus cereus*, *Pseudomonas aeruginosa*, *Clostridium perfringens*, *Clostridium botulinum*, and *Listeria monocytogenes*, often cause serious food poisoning outbreaks as well as sporadic diseases. In *Salmonella* spp., *S.* Typhi, *S.* Typhimurium and *S.* Enteritidis are three of the major serotypes that cause human infection[1]. The latter two serovars are also common species that infect animals[2,3].

The pathogenic strains in *E. coli* include enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), enterohemmorhagic *E. coli* (EHEC) and enteroaggregative *E. coli* (EaggEC). In Taiwan, since 1991, Vibrio parahaemolyticus, *S.* aureus, B. cereus, *Salmonella* spp. and pathogenic *E. coli* have been the prevalent species that cause food-poisoning cases[1].

Because these pathogens cause major food poisoning cases and outbreaks, we have in recent years analyzed molecular types of *Salmonella* Typhimurium, *S.* Typhi, and *S.* Enteritidis. *Salmonella* strains were isolated from patients of food poisoning cases, and animals and their products. *S.* Enteritidis strains obtained from different strain origins in geographically distant areas, such as the USA, were also analyzed and the results were compared with those of the Taiwan isolates. Such molecular typing data would allow us to elucidate the clonal relationship and genetic diversity among the strains of a species, as well as the prevalent subtypes, the epidemic strains that cause human and animal infections, and the strains that are recirculating for food poisoning cases. Such data also are useful for tracing the contamination source when a food poisoning outbreak occurs. Furthermore, the possible strains that are transmitted between different strain origins and geographical areas may also be identified. In this article, we describe the methods that are commonly used for bacteria typing, and give examples of these methods used in subtyping of the *Salmonella* isolates.

METHODOLOGY

Whether conventional biological methods or recently developed molecular methods are used, an ideal typing method should be able to: (a) type the vast majority of strains; (b) show good discriminating ability to recognize a reasonable number of types; (c) generate reproducible results over a long period of time and in different laboratories; (d) readily apply to environment isolates as opposed to the laboratory collections of strains; and (e) be efficient and cost effective.

I. *Conventional Methods of Microbe Typing*

Due to growing awareness of different factors that influence the spread of human and animal pathogens in various environments, conventional typing methods for microorganisms were developed. In conventional methods, the major biological typing techniques are biotyping, phage typing, serotyping and bacteriocin typing (antibiotic resistance typing). These methods have been used to a wide range of microorganism. However, none of these typing methods offers an ideal approach for the subtyping of microbial

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species. The applicability of each of the methods may vary from one species to another. For example, although phage typing has been used with great success, it requires specialized techniques and its application is limited to only certain serotypes. For many species, combination of different methods may be the best approach, but such approach may not be applicable or may take a long time to develop for organisms that have not been well studied before. So far, conventional methods are available for only a limited range of microbial species[4].

II. *Molecular Typing Methods*

Nucleic acids, proteins, and lipopolysaccharide are the only macromolecules that carry information in their sequences and compositions to allow the study of microbial diversity and the development of typing methods[4]. Herein, we would like to focus on the methods for nucleic acid analysis.

A: Analysis of plasmid DNA, ie, plasmid profile and the plasmid restriction endonuclease fingerprint. For such typing analysis, it is not always true that identical plasmid profiles are indicative of an epidemiological relationship of the bacteria. Also, during determination of the sizes of the plasmids or their restriction fragments, considerable size errors can be generated which becomes a problem when attempting to decide on the similarity of two DNA fragments obtained from different laboratories.

B: Analysis of chromosomal DNA. The methods are based on the examination of the DNA fragments obtained from restriction enzyme digestion of chromosomal DNA or polymerase chain reaction (PCR). The commonly used methods are as follows: (a) Pulsed-field gel electrophoresis (PFGE): This method is used for the analysis of restriction enzyme digested chromosomal DNA[5]. After digesting the chromosomal DNA with specific restriction enzyme, the digested DNA are gel electrophoresed and analyzed. (b) Restriction fragment length polymorphism (RFLP): RFLP is generated by the digesting of chromosomal DNA or PCR product amplified with specific primers. RFLP is useful for characterization of several organisms of medical importance. However, depending on the restriction enzyme used, the interpretation of RFLP patterns can be rather difficult because the multitude of chromosomal fragments present and subtle difference may not be observed. (c) Ribotyping: This method is based on the chromosomal DNA digestion followed by electrophoretic separation of the digested DNA with agarose gel, and hybridizating of these DNA fragments with ribosomal RNA or the corresponding gene probes[6]. Ribotyping has been used for both taxonomic purposes and subserogroup characterization of microorganisms belonging to different genera and species. (d) The PCR ribotyping: This method needs the use of specific primers to amplify particular regions of the genome concerned with rRNA and tRNA synthesis. This approach involves the use of PCR to detect polymorphisms in genes or intergenic spacer regions associated with rRNA or tRNA. (e) rep-PCR i.e., the repetitive element sequence-based PCR: This method involves the use of oligonucleotide primers based on short repetitive sequence elements present in all the prokaryotes. These elements appear to be conserved among many members of the Enterobacteriaceae and other bacteria species. (f) Randomly amplified polymorphic DNA fingerprints (RAPD): RAPD is also termed as arbitrarily primed PCR (AP-PCR). This method, although is rapid, may not give reproducible result. (g) Other amplification methods: Examples are: Q β amplification assays, transcript amplification systems (TAS), and Ligase-mediated amplification system, ie, the ligase chain reaction (LCR). (h) Nucleic acid sequencing: This is the most specific and informative method for identification and typing of microorganism. However, it is laborious and expensive.

Among the approaches descried above, PFGE in particular has been widely used in molecular epidemiological investigations of infections caused by a large number of bacterial pathogens, including *S.* Typhi, *S.* Typhimurium and *S.* Enteritidis[7-9]. It has been proposed that PFGE is able to differentiate between clonally related strains (one or two band difference) and strains represent independent clones[5]. Accordingly, when the reproducibility and discriminatory ability are considered, in typing *Salmonella* strains isolated in Taiwan, PFGE is also used as a primary tool.

MOLECULAR TYPING OF THE *SALMONELLA* ISOLATES

I. *Salmonella* Typhimurium

(I) *Analysis of the Salmonella* Typhimurium *Isolates from Food Poisoning Cases*

Due to the importance of *S.* Typhimurium in salmonellosis and food poisoning cases, subtyping of this *Salmonella* serovar has been carried out in many laboratories. For the subtyping of *Salmonella* Typhimurium, methods using phage typing, plasmid profiles[10-13], ribotyping[6,14], IS200 typing[6,15], PCR-ribotyping[16], RAPD[17,18] and PFGE[7,19,20] have been reported. For *S.* Typhimurium, the PFGE method has been shown to be a powerful tool in discriminating the bacterial strains within the same serovar or even within the same phage type[21-23]. Murase et al.[22] has used the PFGE method for epidemiological studies of *Salmonella* infection caused by *S*. Typhimurium, *S*. Thompson and *S.* Enteritidis and found that strains belonging to the same phage type could be discriminated by their PFGE patterns. Thus, to find the prevalent or recirculating stains of *S.* Typhimurium which caused food-poisoning diarrhoea cases in northern Taiwan, we also used PFGE as a primary method. Furthermore, since plasmid profile analysis has been used in epidemiological studies for *S.* Typhimurium infection[12,13] and RAPD method alone has been used for the differentiation of *Salmonella* strains[17,18,24], these methods were also used. Results obtained from the above-described studies indicate that cer-

tain *S.* Typhimurium strains may be the prevalent strains and recirculation of these strains for food poisoning and diarrhoea cases in the same area is possible. The results are shown in Table 1 and briefly described as follows:

When genomic DNAs of 45 randomly selected *S.* Typhimurium strains isolated from food-poisoning cases in northern Taiwan during 1991-1994 were digested with *Xba*I, *Avr*II and *Spe*I, respectively, a total of 26 PFGE combinations of the *Xba*I, *Avr*II and *Spe*I digested DNA patterns were observed. Among the combinations X5A4S4 and X2A2S2 were found to be the major patterns since they contained 13 and 4 strains, respectively. When the 21 plasmid profiles were combined with PFGE patterns, a total of 35 subtypes were found and strains of the subtype of X5A4S4e7 (seven strains) and X2A2S2e7 (three strains) were the prevalent strains. Subtyping by RAPD could not further differentiate these strains grouped by PFGE and plasmid profiles (Table 1). Since strains of the same pattern combinations were isolated from unrelated food-poisoning cases during 1991-1994 in northern Taiwan, these strains

Table 1. Subtyping and isolation dates for the 45 *S. typhimurium* strains

PFGE pattern (*F* value)			Plasmid profile	RAPD typing	No. of strains	Strain no. and date of isolation	Range of isolation	Phage type
*Xba*I	*Spe*I	*Not*I						
X1 (0-606)	A1 (0-500)	S1 (0-791)	e1	b1	1	ISM 01 (09/92)	Taipei	RDNC
X1 (0-606)	A1 (0-500)	S1 (0-791)	e13	b1	1	ISM 22 (06/92)	Taipei	ND
X1 (0-606)	A2 (0-545)	S2 (0-711)	e7	b2	1	ISM 39 (06/91)	Taipei	ND
X2 (0-625)	A2 (0-545)	S2 (0-717)	e2	b2	1	ISM 02 (09/92)	Taipei	DT8
X2 (0-625)	A2 (0-545)	S2 (0-717)	e7	b2	3	ISM 16 (08/94)	Taipei	DT8
						ISM 21 (11/91)	Taipei	ND
						ISM 23 (06/92)	Taipei	DT8
X3 (0-800)	–	–	e2	b2	1	ISM 04 (09/93)	Taipei	ND
X4 (0-667)	–	–	e3	b3	1	ISM 03 (06/93)	Taipei	DT193
X5 (1-000)	A3 (0-842)	S3 (0-977)	e4	b4	1	ISM 05 (11/93)	Taipei	DT104H
X5 (1-000)	A4 (1-000)	S4 (1-000)	e2	b5	2	ISM 07 (11/93)	Taipei	DT104H
						ISM 15 (08/94)	Tanshui	DT104H
X5 (1-000)	A4 (1-000)	S4 (1-000)	e7	b5	7	ISM 25 (06/94)	Taipei	DT104H
						ISM 27 (12/91)	Taipei	ND
						ISM 28 (06/92)	Taipei	ND
						ISM 30 (06/92)	Taipei	ND
						ISM 37 (07/91)	Keehing	ND
						ISM 46 (08/92)	Taipei	ND
						ISM 50 (08/92)	Hsindien	ND
X5 (1-000)	A4 (1-000)	S4 (1-000)	e10	b5	1	ISM 18 (07/94)	Hsindien	U302
X5 (1-000)	A4 (1-000)	S4 (1-000)	e11	b5	1	ISM 19 (06/94)	Tauyang	DT104H
X5 (1-000)	A4 (1-000)	S4 (1-000)	e12	b5	1	ISM 20 (10/94)	Taipei	DT104H
X5 (1-000)	A4 (1-000)	S4 (1-000)	e21	b5	1	ISM 49 (08/92)	Taipei	DT120
X5 (1-000)	A5 (0-909)	S5 (0-905)	e17	b5	1	ISM 33 (08/91)	Tauyang	ND
X5 (1-000)	A5 (0-909)	S5 (0-905)	e18	b5	1	ISM 38 (06/91)	Taipei	U302
X5 (1-000)	A5 (0-857)	S6 (0-744)	e7	b5	1	ISM 41 (09/92)	Taipei	ND
X6 (0-686)	A7 (0-435)	S6 (0-744)	e5	b1	1	ISM 06 (08/93)	Tanshui	U302
X6 (0-686)	A7 (0-435)	S6 (0-744)	e19	b1	1	ISM 44 (08/92)	Taipei	U302
X7 (0-686)	–	–	e6	b6	1	ISM 08 (11/93)	Taipei	DT54
X8 (0-882)	A8 (1-833)	S4 (1-000)	e7	b5	1	ISM 09 (06/93)	Taipei	ND
X8 (0-882)	A4 (1-000)	S4 (1-000)	e7	b5	1	ISM 31 (09/91)	Tauyang	ND
X9 (0-448)	–	–	e7	b7	1	ISM 10 (06/91)	Taipei	DT12
X10 (0-588)	–	–	e8	b1	1	ISM 13 (06/91)	Taipei	RDNC
X11 (0-919)	A9 (0-870)	S7 (0-955)	e2	b5	1	ISM 14 (06/91)	Taipei	ND
X11 (0-919)	A10 (0-909)	S8 (0-933)	e16	b5	1	ISM 29 (08/92)	Taipei	DT120
X11 (0-919)	A11 (0-800)	S9 (0-894)	e7	b5	1	ISM 42 (09/92)	Taipei	ND
X12 (0-667)	–	–	e9	b1	1	ISM 17 (08/94)	Tanshui	ND
X13 (0-706)	–	–	e15	b1	1	ISM 26 (10/91)	Taipei	U302
X14 (0-706)	–	–	e14	b8	1	ISM 24 (06/92)	Taipei	DT120
X15 (0-500)	–	–	e17	b9	1	ISM 32 (09/91)	Taipei	DT12
X16 (0-727)	A12 (0-348)	S10 (0-733)	e7	b1	1	ISM 35 (11/91)	Taipei	U302
X16 (0-727)	A12 (0-348)	S10 (0-733)	e7	b1	1	ISM 36 (07/91)	Taipei	U302
X17 (0-686)	–	–	e20	b1	1	ISM 45 (08/92)	Taipei	U302
X18 (0-588)	–	–	e7	b5	1	ISM 47 (08/92)	Taipei	DT8
X19 (0-611)	–	–	e7	b7	1	ISM 48 (08/92)	Tauyang	DT12

F values are shown in parentheses. Definition of the F value is as described in Methods.
RDNC: reactive but not confirmed. ND: not determined.

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may be the prevalent strains and recirculation of these strains for food-poisoning cases is possible[25]. Similar principle has been used for the study of *Salmonella* Typhi[9] and *S.* Hadar[26]. In the case of *S.* Hadar, subtyping of 73 *S.* Hadar strains isolated from sporadic and epidemic cases in Rome was performed using plasmid profiling, phage typing and antibiotic resistance study, and recirculation of the epidemic strains in the same area has been suggested[26].

(II) *Comparison of Antibiograms of the Salmonella* Typhimurium *Isolates from Humans and from Domestic and other Animals in Taiwan*

Worldwide increase in antimicrobial resistant *Salmonella* infections has been reported during the past decades[27]. In a recent survey from southern Taiwan, *S.* Typhimurium has been shown to be the most resistant species among *Salmonella* species resistant to multiple antibiotics[28]. Most of these *S.* Typhimurium isolates are resistant to ampicillin, chloramphenicol, tetracycline and trimethoprim- sulfamethoxazole. Since *S.* Typhimurium is a common infective pathogen for human and domestical animals and antimicrobial drugs have been commonly used for the control of *Salmonella* infections, it is important to compare the antibiograms for *S.* Typhimurium strains isolated from both origins. We have made such comparison for the 45 human isolates and 87 animal isolates collected from 1990 to 1996 in Taiwan. The antibiotics used were tetracycline (Te), sulfisoxazole (G), ampicillin (Am), chloramphenicol (C), streptomycin (S), trimethoprim- sulfamethoxazole (Sxt), kanamycin (K), gentamicin (Gm), norfloxacin (Nor) and cefoperazone (Cfp). Antibiograms for human and animal isolates are found to be quite similar. The major resistant type for these *Salmonella* strains is TeGAmSC (Table 2). Both the human and animal isolates are highly resistant to first-line antibiotics, such as tetracycline, sulfisoxazole, ampicillin, streptomycin and chloramphenicol. The isolates are also sensitive to fluoroquinolone antibiotics, such as norfloxacin, and the third generation antibiotic of cephalosporin, such as cefoperazone and gentamicin. Between 93% and 100% of the local strains are inhibited by these antibiotics. Also, a significant fraction of these *S.* Typhimurium isolates are multidrug resistant strains. For example, 58.6% of the animal isolates and 68.9% of the human isolates are multidrug-resistant. In conclusion, the antibiograms for human and animal isolates of *S.* Typhimurium are similar. These results may be owing to the fact that Taiwan is a geographically small island and *S.* Typhimurium strains are common infective strains for human and domestic animals. Also, a high fraction of these strains was found to be drug resistant, which may be attributed to the fact that antibiotics are not strictly restricted for use in Taiwan [29].

(III) *PFGE Patterns of Animal Salmonella* Typhimurium *Isolates and the Comparison of them with those of the Human Isolates.*

Table 2. Comparison of the antibiograms for human and animal isolates of *S. typhimurium* obtained in Taiwan

Antibiogram R-types[a]	Percentage in strains tested	
	Human isolates (n=45)	Animal isolates (n=87)
Te G Am S C	48.9	25.3
Te G Am S C Sxt K	6.7	20.7
Te G Am S C Sxt K Gm	4.4	3.4
Te C	0	13.8
G	2.2	5.7
Te G Am S C Sxt K	2.2	1.1
Te G	2.2	2.3
Te G Am S C Sxt	6.7	0
Te G Am S C Cfp	0	3.4
G Am S C Sxt	0	1.1
G Am S Sxt K	0	4.6
Te G Am C	4.4	0
Te Am K	4.4	0
G C	0	1.1
Te G S Sxt	0	2.3
Te G Am Sxt K	0	1.1
Te G Am S C Sxt K Gm Nor	0	2.3
Te	2.2	1.1
Te G S C Sxt K Gm Nor	0	1.1
Te Am G S C Sxt K Nor	0	2.3

a: Antibiotic resistant types were according to NCCLS (1998). Each disk contains: tetracycline (Te) 30 μg, sulfisoxazole (G) 300 μg, ampicillin (Am) 10 μg, streptomycin (S) 10 μg, chloramphenicol (C) 30 μg, trimethoprim-sulfamethoxazole (Sxt) 1.25/23.75 μg, kanamycin (K) 30 μg, gentamicin (Gm) 10 μg, norfloxacin (Nor) 10 μg, and cefoperazone (Cfp) 75 μg, respectively.

S. Typhimurium has been recognized as one of the most prevalent serotypes for *Salmonella* infection in Taiwan [1]. The main source of human infection has been found to be contaminated foods from animal origin[30]. Transmission of *Salmonella* between human and domestic animals has been widely studied because of public health importance[31]. Since the relationship between human salmonellosis and animal infection remains obscure in Taiwan, the chromosomal DNA digestion patterns from the animal isolates were analyzed and compared with those from the human isolates. Chromosomal DNAs from 87 *S.* Typhimurium strains isolated from animals (mostly pigs) were digested with *Xba*I or *Spe*I and electrophoresed to obtain PFGE patterns. The 33 PFGE patterns from the *Xba*I digested chromosomal DNAs are shown in Fig. 1. Strains in two of the *Xba*I digested DNA patterns could be further discriminated by *Spe*I digestion followed by PFGE. This made a total of 38 patterns out of the 87 animal isolates. As the subtyping results from animal isolates were compared with those from the 45 human isolates reported earlier, 14 of the animal isolates and 13 of the human isolates shared a common PFGE pattern combination, i.e., pattern XgSf (or termed as X5S4). Since most of the animal and human strains in pattern XgSf were originally isolated from various areas over different years, and strains of PFGE pattern XgSf (ie. X5S4) may be the most epidemic strains circulating between human and animals sources[3].

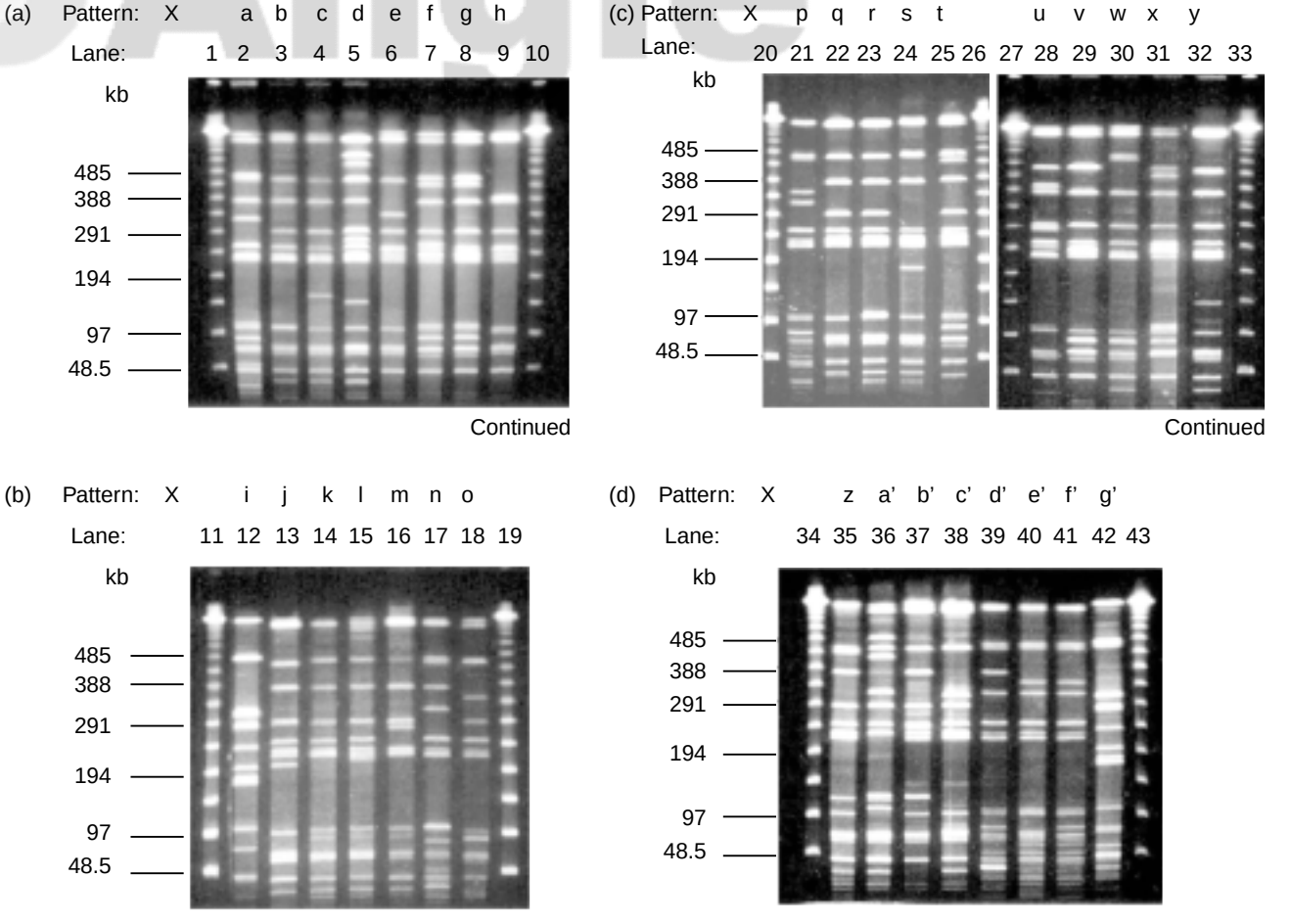

Figure 1. PFGE types for *Xba*I-digested genome DNA of *S. enterica* serovar Typhimurium strains isolated from animals. A total of 87 strains of serovar Typhimurium were analyzed for chromosomal DNA digestion patterns and a total of 33 PFGE types were obtained. (a) Lanes 1 and 10 are the PFGE patterns for marker l ladder. Lanes 2-9 represent the PFGE types Xa-Xh, respectively. (b) Lanes 11 and 19 are the PFGE patterns for marker l ladder. Lanes 12-18 represent the PFGE types Xi-Xo, respectively. (c) Lanes 20, 26, 27 and 33 are the PFGE patterns for marker l ladder. Lanes 21-25 represent the PFGE types Xp-Xt, respectively, and lanes 28-32 represent the PFGE types Xu-Xy, respectively. (d) Lanes 34 and 43 are the PFGE patterns for marker l ladder. Lanes 35-42 represent the PFGE types Xz-Xg0, respectively.

The PFGE data may suggest that some of the sporadic cases for human salmonellosis are the results of the circulation of certain strains between animal and human hosts. Transmission of serovar Enteritidis isolates between human, poultry, broiler chicken and human have been reported in Thailand[2] and United States[32]. Although PFGE patterns for serovar Typhimurium strains isolated from human origin[33] and pigs[34] have been reported in different countries, reports regarding the comparison of these PFGE patterns from different laboratories seem to be absent. Different restriction enzymes and different PFGE conditions used make such comparison difficult.

II. *Salmonella Typhi*

(I) *Use of PFGE as an Epidemiological Tool for Analysis of Salmonella Typhi Strains Isolated from Sporadic Cases in Taiwan*

In many developing countries, such as countries in South America, South-east Asia and Africa, typhoidal fever continues to be an important public health challenge [8,9,35,36]. In Taiwan, in addition to *S.* Typhimurium and *S.* Enteritidis, *S.* Typhi is responsible for a significant part of *Salmonella* infection, although only sporadic cases have been reported in recent years[1,29]. Establishment of molecular typing data for these bacterial species is thus important.

Although various typing methods have been used for the subtyping of *S.* Typhi strains[8,35,37-42], PFGE has proved to be one of the most discriminative methods for the subtyping of *S.* Typhi strains. Thong et al.[8,9,35,36] used the PFGE method for the subtying of *S.* Typhi strains isolated from several southeast Asian countries and found that PFGE was a powerful technique for the analysis of *S.* Typhi strains. Thong et al.[8,9,36] also found that although considerable genetic diversity existed among *S.* Typhi strains, some PFGE patterns might be shared between isolates obtained from different countries, for example, Malaysia, Indonesia and Thailand.

In order to characterize the subtypes of *S.* Typhi, which

cause sporadic disease in Taiwan, 55 isolates of *S.* Typhi obtained from patients unrelated to sporadic cases during 1992-96 were subjected to chromosomal DNA digestion and PFGE. When DNAs of these 55 *S.* Typhi strains were digested with *Xba*I, 41 PFGE patterns were observed. Strains sharing the same *Xba*I digestion pattern could not be further discriminated by PFGE analysis using *Spe*I and *Not*I as digestion enzymes. Thus, considerable genetic diversity existed among the *S.* Typhi isolates. When 12 antibiotics, (i.e. ampicillin, trimethoprim/sulfamethoxazole, erythromycin, norfloxacin, tetracycline, sulphonamide, streptomycin, neomycin, chloramphenicol, kanamycin, cefoperazone and gentamycin) were used to test the antibiotic susceptibility for these *Salmonella* isolates, only 3 antibiogram patterns were obtained and 49 of the 55 *S.* Typhi isolates belong to the same pattern. Such result means that most of the strains are resistant to erythromycin, intermediate to streptomycin and neomycin, but sensitive to the remaining antibiotics used. Phage typing and plasmid profiles are poor in discriminating these strains. For example, 54 of these 55 strains tested showed an absence of the plasmid DNA[43].

Tsen et al.[43] indicate that multiple PFGE patterns, i.e., diversified genetic patterns, are found for *S.* Typhi strains isolated in Taiwan. This has also been observed for *S.* Typhi isolates from other parts of the world, e.g., Malaysia, Indonesia and Thailand[8,9,36]. Considerable genetic diversity has also been found for *S.* Typhi strains isolated in southeast Asia[8]. For example, 46 *Xba*I digestion patterns were found for the 60 strains of *S.* Typhi from Malaysia, and 9 patterns for 10 *S.* Typhi strains obtained in Thailand. Ribotyping studies of *S.* Typhi isolates from USA, Sicily and Malaysia also indicated considerable genetic diversity [44-46]. Diversified genetic patterns for *S.* Typhi may be explained by the recent work of Liu and Sanderson[47], which shows that *S.* Typhi genome has undergone major rearrangements not seen in other *Salmonella* spp. This observation, together with studies by other researchers[8,9,36] and some in vivo genetic variation, suggests a considerable degree of genomic plasticity in the *S.* Typhi genome which may be significant in the virulence of this important human pathogen.

III. *Salmonella Enteritidis*

(I) *Analysis of Salmonella Enteritidis Strains Isolated from Food-poisoning Cases in Taiwan by PFGE, Plasmid Profile and Phage Typing.*

S. Enteritidis may cause non-typhoid salmonellosis and is a very important food pathogen[1,48]. Human infections with *S.* Enteritidis have been increasing worldwide since 1980. Epidemiological studies have implicated consumption of eggs and egg products[2]. In recent years, *S.* Enteritidis has emerged as a major serovar in Taiwan[1]. Because of the increasing role of *S.* Enteritidis in *Salmonella* infections in Taiwan, establishment of the molecular typing data for this *Salmonella* species is important.

For the subtyping of *S.* Enteritidis, methods of phage typing and PFGE[2,21,23, 48,49,50], plasmid profile[51], IS 200 typing[21], ribotyping[21,52] and random amplified polymorphic DNA[24,49], etc. have been reported. Thong et al.[23,48] used the PFGE method to trace the clonal relationship of *S.* Enteritidis strains which caused foodborne outbreaks. They found a highly clonal nature of pathogenic strains of *S.* Enteritidis. On subtyping *Salmonella* strains, it has been reported that PFGE allows strain discrimination for some *Salmonella* serotypes, including *S.* Enteritidis[21-23,48,49]. Thus, on performing the epidemiological analysis of the diarrhea case-associated strains of *S.* Enteritidis collected between 1991 and 1997, PFGE method was also used as a primary method. Furthermore, since plasmid profiles have been used for the analysis of *S.* Enteritidis[51,53,54] and phage typing has been commonly used method of *Salmonella* typing since 1950[17], these methods were also used in the study. The results indicate that there is a clear association with PT4 phage type in the food poisoning related isolates of *S.* Typhimurium obtained in Taiwan. These *Salmonella* strains are genetically very similar or related as assayed by the PFGE method. A brief description for the subtyping of these *S.* Enteritidis strains isolated in Taiwan are given below.

63 *S.* Enteritidis strains isolated from patients suffering from food borne poisoning during 1991-97 were collected and subjected to PFGE, plasmid analysis and phage typing. For PFGE, *Xba*I, *Spe*I and *Not*I restriction enzymes were used for chromosomal DNA digestion. The results showed that, for these 63 *Salmonella* strains, 10 PFGE pattern combinations were found. Of these, pattern X3 S3 N3 was the major subtype since 46 strains isolated from different locations at different times during 1991-97 showed this PFGE pattern. Thus, limited genetic diversity was found for those *S.* Enteritidis strains. Plasmid analysis showed only 3 plasmid profiles and phage typing showed that most of the *Salmonella* strains were of the phage type PT4 (Table3). Thus, most of the *S.* Enteritidis strains circulating in Taiwan are of very similar genetic types or are highly related. The results also showed that strains of PFGE pattern X3 S3 N3 are the prevalent and recirculating strains of *S.* Enteritidis which caused food-poisoning cases in Taiwan during 1991-97[55].

It should be mentioned that pattern X3 S3 N3 has also been found among *Salmonella* strains obtained from countries near Taiwan, such as Philippine (strains ISE 34, 35, 36 and 37) and Malaysia (strains ISE 27 and 32). Such results raise the question of whether PFGE type X3 S3 N3 is also the major subtype for strains isolated in other countries, especially countries of geographically distant areas, and for strains from origins other than human. In a separate study, we have used the same PFGE method to analyze 77 strains of *S.* Enteritidis obtained from veterinary origins in USA, a country geographically distant from Taiwan. The results show that PFGE pattern X3 S3 N3 is one of the only two co-shared patterns among a total of 32 PFGE patterns found for the Taiwan and US isolates.

Table 3. Plasmid profiles, pulsed field gel electrophoresis (PFGE) patterns and phage types for *Salmonella enteritidis* strains isolated from patients with food-borne diarrhoea in Taiwan

PFGE pattern						
*Xba*I	*Spe*I	*Not*I	Plasmid profile	Strain no. and date of isolation	Range of isolation	Phage type*
X1	S1	N1	P1	ISE 01 (91)	Hua-lan	PT4
X2	S2	N2	P1	ISE 02 (92)	Taipei	PT4
X2	S2	N2	P2	ISE 12 (96)	Taipei	–
				ISE 29 (97)	Chang-Hua	PT27
				ISE 53,54,55,56,57 (97)	Chang-Hua	RDNC
X3	S3	N3	P1	ISE 03 (92)	Hua-lan	PT4
				ISE 10 (95)	Tau-Yuan	PT4
				ISE 11 (96)	Taipei	–
				ISE 13 (96)	Tau-Yuan	PT7
				ISE 14, 15 (96)	Tau-Yuan	PT4
				ISE 16,17,18,19 (96)	Taipei	PT4
				ISE 20,21,22 (97)	Tau-Yuan	PT4
				ISE 23 (97)	Taipei	PT4
				ISE 25 (97)	Tau-Yuan	PT4
				ISE 24 (97)	Taipei	PT4a
				ISE 26 (97)	Taipei	–
				ISE 27,32 (97)	Malaysia → Taipei	PT4
				ISE 28 (97)	Tau-Yuan	PT4
				ISE 31 (97)	Taipei	PT4
				ISE 33 (97)	Kao-Shiung	PT4
				ISE 34,35,36,37 (7/30,92)	Philippine	PT4
				ISE 38,39,40,41,42 (8/28,95)	Taipei	PT4
				ISE 43,45,46,47 (9/24,96)	Yi-Lan	PT4
				ISE 48,49,50,51,52 (7/25,97)	Hua-Lein	PT4
				ISE 58,59,60,61,62 (10/22,97)	Chung-Li	PT4
				ISE 30 (97)	Taipei	–
X3	S7	N3	P1	ISE 63 (97)	Peng-Hu	PT4
X4	S4	N4	P1	ISE 04 (93)	Hua-lain	PT34
X5	S4	N4	P1	ISE 05 (94)	Hua-lain	PT8
X6	S5	N5	P1	ISE 06 (95)	Taipei	PT4
X7	S2	N2	P2	ISE 07 (95)	Taipei	PT13
X8	S6	N6	P1	ISE 08 (95)	Tau-Yuan	PT8
X9	S6	N7	P3	ISE 09 (95)	Taipei	PT8
X10	S3	N3	P1	ISE 44 (96)	Yilan	PT4

* Only strains of ISE 01, 02, 03, 04, 05, 06, 07, 08, 09, 10, 13, 14, 15, 16, 17, 20, 23, 24, 25, 26, 27, 29, 31, 33, 34, 38, 43, 45, 46, 48, 53, 58 and 63 were deterninted for their phage types.

DISCUSSION

This article briefly describes the general methods used for bacteria subtyping. These methods include the conventional methods, such as biotyping, phage typing, serotyping, bacteriocin typing (antibiotic resistance typing) and the molecular methods, such as plasmid profile, plasmid restriction endonuclease fingerprints, PFGE, ribotyping, RFLP, PCR-ribotyping, rep-PCR, RAPD or AP-PCR etc. For many bacterial species, combination of different methods or selection of the most discriminative methods is usually required to identify particular strains. Also, while performing these typing methods, especially the molecular typing methods, defined critical condition should be followed to assure reproducible results. For example, it is known that changes in parameters, like temperature, voltage, agarose concentration and ionic strength will affect the mobility of different sizes of DNA. Changes of any parameter may affect resolution adversely. Accordingly, it may be the reason that although the molecular typing data for some bacteria have been reported in different countries, reports regarding the collection of these typing data from different laboratories and comparison of them seem to be absent. Such comparison will not be possible until standardized protocol and interpretation method are developed[8].

Several conclusions can be made from the works for the molecular typing of *Salmonella enterica* serovars Typhimurium, Typhi, and Enteritidis isolated in Taiwan. (1) Although considerable genetic diversity is found in some bacteria, such as *S.* Typhimurium, the most disseminated or recirculated strains can be identified. Similarly, multiple PFGE patterns, i.e., diversified genetic patterns, are found for *S.* Typhi strains isolated in Taiwan. On the other hand, *S.* Enteritidis strains isolated in Taiwan seem to be genetically similar, and strains of a specific subtype may be the most prevalent as well as recirculating strains. (2) In *S.* Enteritidis

strains, certain subtypes are shared by strains isolated in Taiwan and strains obtained from geographically far distant areas, like USA. Strains of these subtypes may be the most disseminated and epidemic strains. Whether these strains are more virulent or resistant to the changes of environments need to be further investigated. (3) Strains of the same subtype have been found in food isolates, animal isolates and clinical isolates from humans suffered from salmonellosis. Thus, transmissions of the strains among foods, humans and animals is possible. Finally, it should be mentioned that studies with more strains collected in longer periods from different origins are necessary to further strengthen the above conclusions.

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台灣分離鼠傷寒、傷寒及腸炎沙門氏桿菌血清型菌株之分子分型

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摘　要

鼠傷寒、傷寒及腸炎血清型沙門氏桿菌是重要之食品病原菌，可造成人類疾病及（或）動物之感染，欲瞭解個別血清型內菌株之種源關係和找出分佈最廣及食品中毒案件中重複出現之菌株，以及這些菌株在不同分離源及地區之可能轉移，我們利用噬菌體分型，抗生素圖譜及分子分類之方法，如質體圖譜（plasmid profile）、脈衝式電場膠體電泳（PFGE）及隨機擴大多型性DNA分析（RAPD），以鑑定沙門氏菌之次分型。結果發現鼠傷寒沙門氏菌或傷寒沙門氏菌血清型內之各菌株，其基因型有相當的多樣性。然而，腸炎沙門氏菌血清型的菌株有高度的基因相似性。此外，鼠傷寒及腸炎沙門氏菌血清型中之食品中毒案件中最常見之菌株，也可被確認出，這些菌株在不同來源及地區間，可能是優勢菌株，而不同地區、不同分離源間菌株的轉移是可能的。

關鍵詞：鼠傷寒、傷寒及腸炎血清型沙門氏桿菌，分子分型