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Genome fingerprinting of *Salmonella typhi* by pulsed-field gel electrophoresis for subtyping common phage types

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SUMMARY

The genomic DNA of 39 strains of *Salmonella typhi* isolated from local residents and patients who had visited countries in the Asian region was analysed for restriction fragment length polymorphisms (RFLP). Pulsed-field gel electrophoretic (PFGE) analysis of *Xba* I- and *Spe* I-generated genomic restriction fragments established 22 PFGE types whereas phage typing differentiated the 39 isolates into 9 distinct phage types. This study showed that PFGE is more discriminatory than phage typing as it is capable of subtyping *S. typhi* strains of the same phage types. Genetic relatedness among the isolates was determined. Seven major clusters were identified at S_{ABs} of $> 0{\cdot}80$ and the remaining 13 isolates were distributed into minor clusters which were related at S_{ABs} of less than 0·80. In conclusion, PFGE analysis in conjunction with distance matrix analysis served as a useful tool for delineating common *S. typhi* phage types of diverse origins from different geographical locales and separated in time.

INTRODUCTION

Typhoid fever is caused by *Salmonella typhi* isolates which can be either indigenous in a community or imported. Currently, sub-division of *S. typhi* is based on phage typing [1]. Phage typing may be of limited value if few phage types represent the majority of strains present in a particular locale [2]. Newer molecular methods for the characterization of *S. typhi* such as ribotyping have been reported by Altwegg and colleagues [3] to be useful for further differentiation of common phage types. However, its use may be limited to those laboratories where the preparation of rRNA gene probes, Southern blots and hybridization using radioactive or non-radioactive approaches are available. Therefore, molecular techniques that are cheaper, less laborious and can provide discrimination within common phage types are needed.

Restriction endonuclease analysis (REA) of genomic DNA digested with high frequency cleavage enzymes was found to be of limited use in further

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differentiation of *S. typhi* strains within common phage types [4]. Alternatively, REA of genomic DNA can be achieved by cleavage with rare cutting restriction enzymes and resolution of restriction fragments by pulsed-field gel electrophoresis (PFGE). This approach has been used successfully for epidemiological investigations of outbreaks caused by pathogens such as *Shigella* spp. [5], *Bordetella pertussis* [6] and methicillin-resistant *Staphylococcus aureus* [7]. As PFGE generates fingerprints of each strain, valuable data such as genome sizes and maps can be readily derived. PFGE has also been shown to be a relatively sensitive method for establishing clonal relationships among epidemic strains of pathogens [8, 9]. In the present study, the potential utility of pulsed-field gel electrophoresis as a tool for investigating the epidemiology of *S. typhi* infections was assessed. The results were analysed to reveal the clonal relationships of *S. typhi* phage types.

MATERIALS AND METHODS

Bacterial isolates

The 39 *S. typhi* studied had been isolated from patients between the ages of 2–61 years old and who had been admitted to 1 of 7 different hospitals in Singapore between July 1989 and January 1993. Thirty-one *S. typhi* strains were isolated from blood, six from stool specimens and one each from pus and urine specimens.

Phage typing

All the *S. typhi* isolates were phage-typed according to the method of Anderson and Williams [10]. The phages were used at the routine test dilution, that is, the highest dilution that produced confluent lysis of the homogenous type strain. The temperature of incubation was 38·5 °C.

Preparation of genomic DNA

S. typhi isolates were grown in 100 ml of LB broth with shaking at 200 rpm for 24 h at 37 °C. Cells were pelleted by centrifugation at 6000 g for 10 min at 4 °C. Pelleted cells were resuspended in 3 ml of solution I (10 mM-Tris-HCl [pH 7·5]; 1 M-NaCl). One ml of the suspension was then mixed with 2 ml of 1% low melting point agarose (Sea Plaque LMP, FMC). The mixture was dispensed into slots (10 × 6 × 1 mm) and allowed to solidify for 10 min at 4 °C. The agarose blocks were transferred into 10 ml of lysis solution (6 mM-Tris-HCl; [pH 7·6]; 100 mM-EDTA [pH 7·5]; 1 M-NaCl; 0·5% Brij-58; 0·2% sodium deoxycholate; 0·5% sodium lauryl sarcosine; 10 mg lysozyme; 10 U RNase) and incubated overnight at 37 °C. The lysis solution was removed and replaced with 10 ml of solution II (0·5 M-EDTA, [pH 9·0]; 1% sodium lauryl sarcosine; 50 μg/ml proteinase K) and incubated at 55 °C for 48 h. The blocks were washed for two 1-h intervals at 37 °C in 1 × TE (10 mM-Tris; 10 mM-EDTA, pH 7·4) and stored in 1 × TE at 4 °C.

Digestion by restriction endonuclease and PFGE

One DNA plug prepared as described above was equilibrated in 150 μl of restriction endonuclease buffer for 30 min on ice as recommended by the manufacturer. The buffer was then aspirated, the tube filled with fresh buffer and digestion of the DNA was carried out with 10 U of the restriction endonuclease. The reaction mixture was incubated overnight at 37 °C. After digestion, the plugs

were loaded into slots of a gel consisting of 1 % agarose in 0·5 × TBE (45 mM-Tris, 45 mM boric acid, 1·0 mM-EDTA [pH 8·0]). Electrophoresis of digested DNA fragments was carried out by PFGE using a contour-clamped homogenous electric field (CHEF) apparatus with a hexagonal electrode array (CHEF-DR III; Bio-Rad). PFGE was performed at 14 °C for 27 h at 6 V/cm with a pulse time from 1–60 s and angles were set at 120 °C. Genomic DNA of *Rhodobacter sphaeroides* 2·4·1 digested with *Ase* I was used as the molecular weight standard [11]. The DNA restriction patterns were photographed with a Polaroid MP4 camera with a red filter and type 55 Kodak film.

Analysis of PFGE data

PFGE profiles were analysed by an automated Dendron program (Solltech Inc., Iowa, USA) [12]. The PFGE profiles were digitized into the Dendron database with a Sharp scanner. Bands were automatically identified and were classified between 0 (no bands) and 3 (highest intensity). The Dendron program then computed the similarity coefficient (S_{AB}) for each pair of strains on the basis of position and intensity of the bands in the profiles, to generate a matrix of similarity coefficients. The profiles were checked and when necessary manually corrected. Dendrograms based on S_{AB} were then generated for the 39 isolates by using the unweighted group pair [13] method.

RESULTS

Phage typing

Nine phage types (PTs) were represented among 39 *S. typhi* clinical isolates. Six exhibited PT UVS1 pattern. PTs B1, D1, D2, E1 and M1 each had 5 isolates. PTs 28 and UVS4 were each comprised of 3 isolates whereas 2 isolates were classified as PT UVS2 (Table 1).

Pulsed-field gel electrophoresis

Four restriction endonucleases (*Xba* I, *Spe* I, *Nhe* I and *Avr* II) which recognize genomic sequences containing the rare tetranucleotide CTAG were screened for their digestion patterns. Both *Xba* I and *Spe* I produced clearly resolvable fragment patterns that were easily interpreted. Digestion with *Xba* I produced fingerprints consisting of 16–21 DNA fragments that ranged in size from 5–660 kb. Reproducibility of the *Xba* I generated PFGE profiles was determined by testing of the same isolate on three separate occasions. Stability of PFGE profiles was also confirmed by the presence of identical restriction patterns generated by cells from a single isolate that had undergone nine serial passages.

The relatedness of PFGE profiles between two isolates was scored by the coefficient of similarity [14]. The value (F) reflected the proportion of shared fragments in the two genomes. On the basis of F values generated, a total of 22 major PFGE types were established by *Xba* I digestion of genomic DNA from the 39 isolates. Within each major PFGE type, there exist strains which were classified as clonal variants and these were indicated by a numerical suffix. Clonal variants were found to differ from the major PFGE profile by up to three DNA fragments and they generally expressed F values above 0·8. PFGE profiles of *Spe* I-digested genomic DNA showed the presence of 21–25 fragments that ranged

Table 1. *Geographic distribution of* S. typhi *isolates*

Strain number	Isolate designation	Date of isolation	Classification	Country of origin
1	SINM 13/92	25/2/92	Local	Singapore
2	SINM 20/92	16/3/92	Imported	Indonesia
3	SIN 21/91	17/11/92	Imported	India
4	SINM 21/92	20/3/92	Imported	Philippines
5	SIN 23/92	27/4/92	Local	Singapore
6	SINM 25/92	30/4/92	Imported	India
7	SINM 2/92	9/11/92	Imported	India
8	SINM 22/91	19/2/91	Local	Singapore
9	SINM 77/91	22/11/91	Local	Singapore
10	SIN 8/91	6/3/91	Local	Singapore
11	SIN 14/91	4/7/91	Local	Singapore
12	SIN 5/91	29/1/91	Local	Singapore
13	SINM 12/93	22/2/93	Imported	Indonesia
14	SIN 18/91	30/1/91	Imported	India
15	SINM 62/91	26/9/91	Imported	India
16	SIN 73/90	20/5/90	Imported	Bangladesh
17	SINM 59/91	30/8/91	Imported	Vietnam
18	SIN 1/91	3/1/91	Imported	India
19	SIN 66/89	25/7/89	Imported	India
20	SINM 17/92	9/4/92	Imported	India
21	SINM 12/92	25/12/92	Imported	India
22	SINM 5/92	20/1/92	Imported	India
23	SINM 24/92	28/4/92	Imported	India
24	SINM 19/92	19/4/92	Imported	India
25	SINM 84/91	18/12/91	Local	Singapore
26	SINM 14/91	25/1/91	Imported	Bangladesh
27	SIN 1/90	2/1/90	Local	Singapore
28	TSINM 1/93	8/1/93	Imported	Indonesia
29	SINM 40/92	2/9/92	Imported	Indonesia
30	SINM 26/92	30/6/92	Imported	Thailand
31	SINM 13/91	16/1/91	Imported	Philippines
32	SINM 16/92	11/3/92	Imported	Philippines
33	SINM 1/93	5/1/93	Imported	Philippines
34	SSINM 13/93	24/2/93	Imported	Indonesia
35	SINM 31/92	4/7/92	Imported	Indonesia
36	SINM 33/92	13/7/92	Imported	Indonesia
37	SINM 10/92	7/2/92	Local	Singapore
38	SINM 11/92	9/3/92	Local	Singapore
39	SINM 15/92	9/3/92	Local	Singapore

in size between 5–1105 kb. Based on *Spe* I-generated restriction patterns, 22 major PFGE profiles were evident amongst the 39 isolates. The distribution of the isolates in each of the *Spe* I PFGE groups was in general agreement with those determined by *Xba* I digestion.

Comparison of PFGE and PT

Within the group of six isolates that showed PT UVS1 pattern, there were six different PFGE profiles (types 1–6) (Fig. 1, lanes B to G). PFGE profiles of the isolates were heterogeneous. For the three PT UVS4 isolates, three distinctly different PFGE profiles (types 7–9) were evident. The two PT UVS2 isolates were differentiated into two closely related variants by both *Xba* I and *Spe* I digestion.

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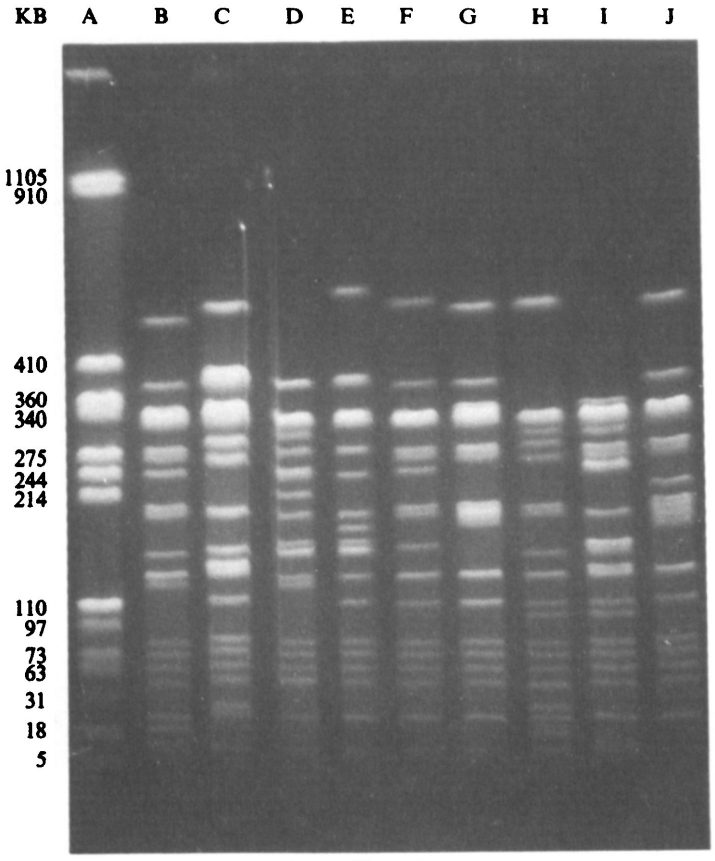

Fig. 1. Pulsed-field gel electrophoresis of *Xba* I digested *S. typhi* strains belonging to UVS1 (lanes B–G) and UVS4 (lanes H–J) phage types. Molecular weight of DNA fragments were obtained from *Ase* I digested *R. sphaeroides* genomic DNA.

Of the five PT M1 isolates examined, three had identical PFGE profiles (Fig. 2, lanes D to F). The remaining two PT M1 isolates had closely related *Xba* I profiles that differed by two band differences from each other (Fig. 2, lanes B and C) and from the rest. Compared with the three PT M1 isolates with PFGE type 11 profile generated by *Xba* I digestion, isolate SINM 84/91 with type 11a profile and isolate SINM 14/91 with type 11b profile were found with two additional DNA fragments in the 170–210 kb region.

Each of the five isolates with PT E1 were grouped as PFGE profile type 12, four of which are shown in Fig. 2 (lanes G–J). Of the five PT D2 isolates, two PFGE profiles were evident. One pair had a PFGE type 13 profile whereas the other three isolates yielded an identical PFGE profile designated as type 14. Isolates with profile type 13 differed from isolates with type 14 profile by up to six DNA fragments.

Amongst the five PT D1 isolates, two had identical PFGE profiles (type 17) (Fig. 3, lanes F and G). Isolate SIN 5/91 had up to three DNA band differences (Fig. 3, lane H) from these two type 17 isolates. Two other PT D1 isolates (types 18 and 19) were found with PFGE profiles that differed from isolates with type 17 profile by up to five and six DNA fragments respectively (Fig. 3, lanes I and J)

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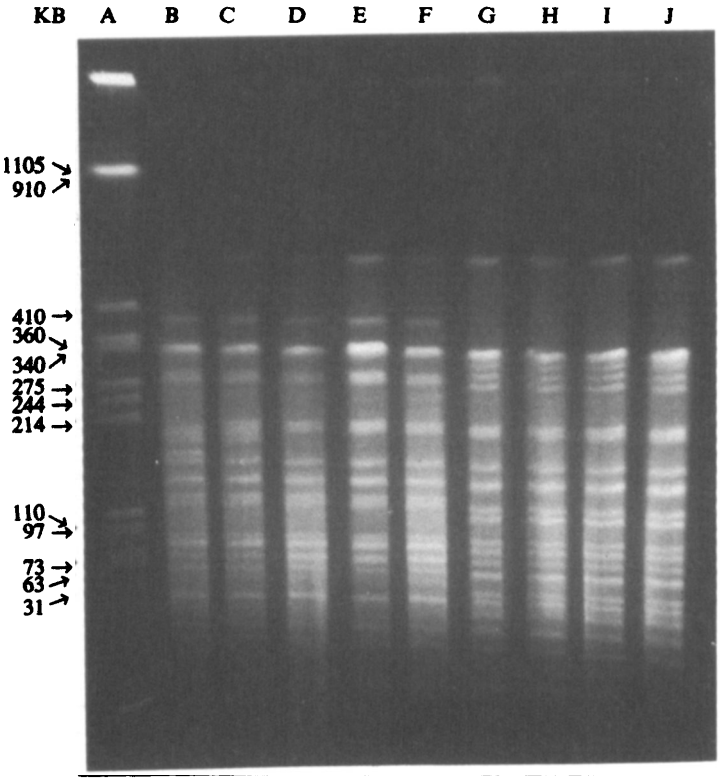

Fig. 2. Agarose gel showing *Xba* I digestion patterns of *S. typhi* M1 (lanes B–F) and E1 (lanes G–J). Lane A, genomic DNA from *R. sphaeroides* digested with *Ase* I.

For the five PT B1 isolates, three were found with the same PFGE profile (type 20). PFGE profiles of two of these are shown in Fig. 3 (lanes B and C) while the remaining two PT B1 isolates had clearly different PFGE profiles (types 21 and 22) (Fig. 3, lanes D and E). The latter two isolates differed from the three type 20 isolates by up to five DNA bands.

Analysis of genetic relationships determined by PFGE

An analysis of *Xba* I profiles of the 39 isolates was carried out using the computer assisted automated Dendron system to determine their genetic relatedness. A dendrogram based on S_{AB} of each individual isolate was generated [Fig. 4]. As suggested by Hellstein and colleagues [15], we used S_{AB} 0·80 as an arbitrary threshold for genetic similarity and seven clusters could be identified at S_{AB} of > 0·80 [Fig. 4]. Three clusters were each comprised of five isolates (cluster a, b and c), one cluster contained four isolates (cluster d), one cluster three isolates (cluster e). Two clusters were each found with two isolates (clusters f and g) [Fig. 4]. The remaining 13 isolates were related to all other isolates at S_{AB} of less than 0·80 [Fig. 4].

DISCUSSION

Differentiation of strains involved in *S. typhi* infections depends on accurate epidemiological markers. The most common method used to date for the

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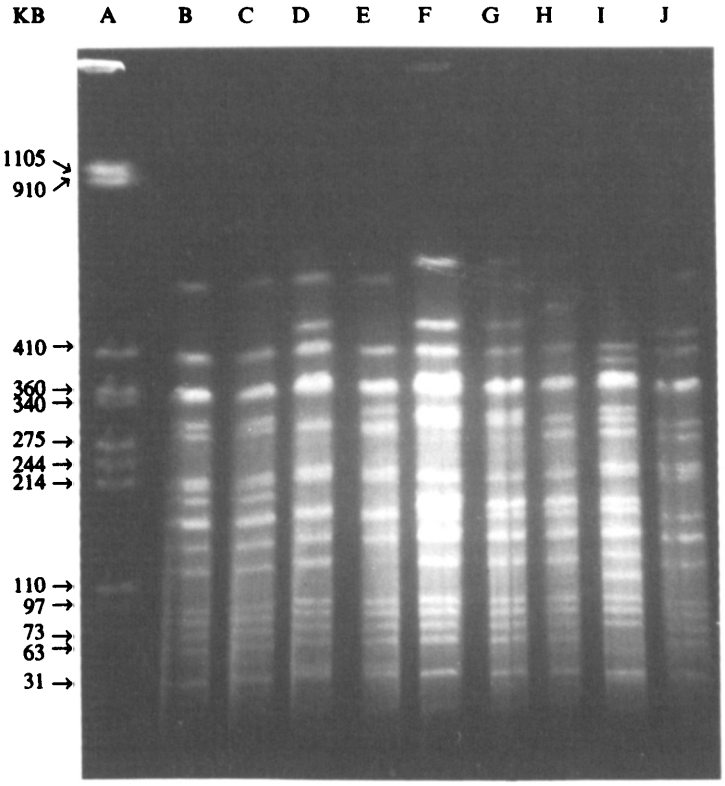

Fig. 3. Pulsed-field gel electrophoresis of *Xba* I digested *S. typhi* strains belonging to B1 (lanes B–E) and D1 (lanes F–J) phage types. Molecular weight of DNA fragments were obtained from *Ase* I digested *R. sphaeroides* genomic DNA.

demonstration of epidemiologic associations among isolates of *S. typhi* has been phage typing. However, phage typing has certain limitations. For instance, it may be of limited value if a common phage type or a limited number of phage types predominate [16]. Changes in lysis patterns can occur as a result of acquisition or loss of lysogenic phages [17].

In recent years, newer typing techniques such as multilocus enzyme electrophoretic analysis [18], ribotyping [19], plasmid profile analysis [20] and IS200 fingerprinting [21] have been developed to discriminate between isolates of *S. typhi*. Reeves and colleagues [18] showed that multilocus enzyme electrophoretic analysis could not differentiate *S. typhi* strains of different phage types and concluded that a single clone was responsible for *S. typhi* infections despite the widespread geographical origins of the strains. This observation was supported by Maher and colleagues [16] who found chromosomal and plasmid profiling to be of limited value in differentiating a collection of 141 *S. typhi* of known phage types. Similarly, genomic fingerprinting by IS200 was found to be less discriminatory when compared to phage typing. Ribotyping based on rRNA gene restriction patterns was shown by several groups [3, 22] to be effective in subtyping *S. typhi* strains that either belong to common phage types or were untypable. However, ribotyping is more costly, technically more complex and the discrimination

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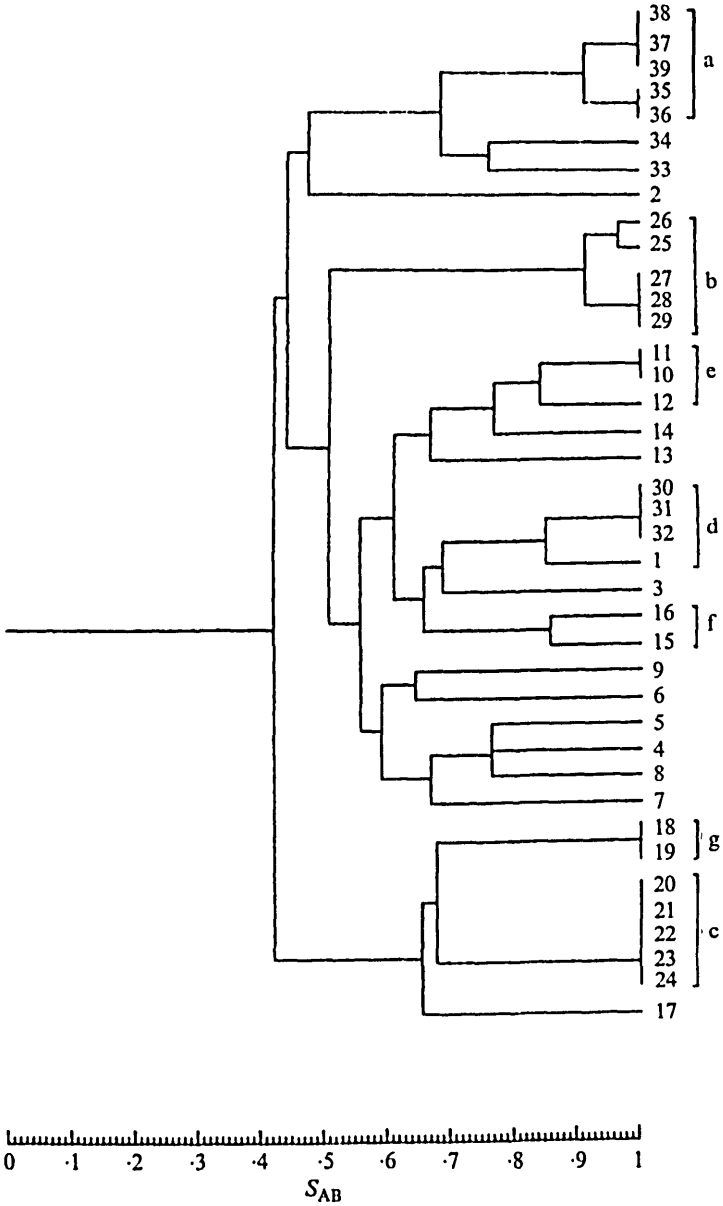

Fig. 4. Genetic relationships among the 39 *S. typhi* isolates. The dendogram was generated by a computer assisted program of PFGE profiles. A S_{AB} value of 0·80 was considered the threshold for significant degree of similarity. The strain numbers are shown in Table 2.

achieved is largely dependent on the choice and or number of restriction enzymes used. Moreover, several recent studies have indicated that ribotyping is less discriminatory than PFGE analysis [23–25].

Thus, the discriminatory power of typing methods used in epidemiological investigations is critical to conclusions drawn regarding infections caused by *S. typhi* strains. It is important to establish whether strains involved in outbreaks are homogeneous or heterogeneous as it will help in the identification of epidemio-

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logical relationships of strains between patients, carriers and possible sources of infection. In this study, the 39 *S. typhi* isolates were differentiated into 9 different phage types (PTs) but 22 different pulsed-field gel electrophoretic (PFGE) types. The higher discriminatory power of PFGE analysis must be interpreted with reference to the extent of DNA band differences between strains and their correlation with epidemiological data. Analysis of genetic relationships among the 39 strains indicated that 7 major clones/lineages (clusters a–g) (Fig. 4) interspersed with 13 minor genotypes have spread through this region of the world involving India, Vietnam, Thailand, Singapore, Philippines, Bangladesh and Indonesia.

A high degree of correlation between phage typing and PFGE analysis was observed for five PT E1 isolates. These five isolates were grouped in cluster C and the genome size of each was 2995 kb. Both typing methods correlated well with the epidemiological data and showed that this clone was imported from India.

The PFGE profiles of five PT M1 isolates displayed closely related patterns. Of the three isolates with identical PFGE type 11 profile from cluster b, two were acquired from Indonesia and one was a local isolate. The similarity of PFGE profiles, phage types and high level of genetic relatedness of the three epidemiologically unrelated strains (isolates SIN 1/90, SINM 40/92 and TSINM 1/93) reflect the spread of a single clone over a large geographical area and over a period of 3 years. The genome size of each of the three PT M1 isolates was 3269 kb. Two other isolates (SINM 84/91 and SINM 14/91) with closely related PFGE patterns to type 11 (difference of only 2–3 DNA fragments) were considered as variants of this common clone circulating in Asia. The genome size of isolates SINM 84/91 and SINM 14/91 were 3580 and 3636 kb respectively. Both the epidemiological and PFGE data supported the conclusion that a major clone (type 11) and two clonal variants (type 11a and 11b) were responsible for the infections that occurred in the five patients instead of a circulating common clone of PT M1.

Two PT D2 strains isolated from a brother and sister returning from Indonesia had the same PFGE type 13 profile (genome size of 3445 kb). The two type 12 PT D2 strains were closely related but not identical to a group of three isolates with type 14 PFGE profile (genome size 3377 kb). The high level of genetic relatedness again indicated that the five isolates were clonal variants arising from a single clone which had predominated over a wide geographical area.

In contrast to the close genetic relatedness of PT M1 and D2 strains, a more distant relationship was observed amongst some strains of PT B1 and D1. Three PT B1 strains with type 20 PFGE profile were imported from the Philippines and Thailand in 1991 and 1992. These 3 strains displayed low genetic relatedness with 2 other PT B1 strains isolated in 1993 from patients returning from Indonesia and the Philippines. The data clearly confirmed that the 2 strains isolated in 1993 were of different clonal origin to the 3 isolated during 1991 and 1992. For the 5 D1 isolates, analysis of PFGE showed that 2 of the 3 D1 strains isolated from 3 patients residing in a mental institution were identical and the third D1 strain was a clonal variant. These 3 strains were not closely related to the 2 strains isolated from patients returning from India and Indonesia. Based on phage typing, one would have concluded that a common clone of PT D1 had persisted in three different countries at different periods of time. Similarly, the 3 PT 28 strains were of 2 different clonal origins. The 2 strains isolated from patients returning from

Table 2. *Genotypic characteristics of* S. typhi *phage types*

Strain number	Isolate designation	Phage types	PFGE types *Xba* I	PFGE types *Spe* I	Plasmids (kb)	Total genomic size (kb)
1	SINM 13/92	UVS1	1	1	ND	3241
2	SINM 20/92	UVS1	2	2	ND	3011
3	SIN 21/91	UVS1	3	3	ND	2935
4	SINM 21/92	UVS1	4	4	ND	3195
5	SIN 23/92	UVS1	5	5	ND	3271
6	SINM 25/92	UVS1	6	6	ND	3420
7	SINM 2/92	UVS4	7	7	ND	3077
8	SINM 22/91	UVS4	8	8	60	3205
9	SINM 77/91	UVS4	9	9	60	3472
10	SIN 8/91	D1	17	18	ND	3269
11	SIN 14/91	D1	17	18a	ND	3269
12	SIN 5/91	D1	17a	19	60	3279
13	SINM 12/93	D1	19	20a	ND	3100
14	SIN 18/91	D1	18	20	60	3208
15	SINM 62/91	UVS2	11	10a	60	2742
16	SIN 73/90	UVS2	10	10	60	3175
17	SINM 59/91	28	16	17a	ND	3399
18	SIN 1/91	28	15	17	ND	2832
19	SIN 66/89	28	15	17	ND	2832
20	SINM 17/92	E1	12	14	ND	2995
21	SINM 12/92	E1	12	14	ND	2995
22	SINM 5/92	E1	12	14	ND	2995
23	SIN 24/92	E1	12	14	ND	2995
24	SINM 19/92	E1	12	14	ND	2995
25	SINM 84/91	M1	11a	12	60	3580
26	SINM 14/91	M1	11b	13	ND	3636
27	SIN 1/90	M1	11	11	ND	3269
28	TSINM 1/93	M1	11	11a	ND	3269
29	SINM 40/92	M1	11	11	ND	3269
30	SINM 26/92	B1	20	15	ND	2938
31	SINM 13/91	B1	20	15	ND	2938
32	SINM 16/92	B1	20	21	ND	2938
33	SSINM 1/93	B1	21	22	ND	3157
34	SSINM 13/93	B1	22	15	ND	2874
35	SINM 31/92	D2	13	15	60	3445
36	SINM 33/92	D2	13	15	60	3445
37	SINM 10/92	D2	14	16a	ND	3377
38	SINM 11/92	D2	14	16	ND	3377
39	SINM 15/92	D2	14	16	ND	3377

* a and b denote clonal variants.

India in 1989 and 1991 were different to the strain isolated from a patient returning from Vietnam in 1991.

The lack of genetic relatedness among PTs UVS1, UVS2 and UVS4 was clearly reflected at S_{AB} values of less than 0·8. These isolates were of diverse clonal origin and they were derived from different geographical locales.

PFGE analysis is a technique that involves the evaluation of whole-cell DNA (genomic together with plasmid DNA). From our studies, it was shown that only 9 of the 39 isolates carried a 60 kb plasmid (Table 2). The presence of a single plasmid in a strain would contribute a single band difference when compared to a plasmid-free strain on the assumption that the plasmid contained no more than one

restriction site for the enzyme used. Acquisition of a plasmid by any strain derived from a single clone would then generate a closely-related clonal variant. In the present investigation, two closely related clonal variants (isolates SIN 14/91 and SIN 5/91) differed not only in the plasmid content but significant differences were observed in larger DNA fragments. In most instances, differences in PFGE profiles generated were not influenced to a large extent by the presence of the 60 kb plasmid. The variants tended to exhibit differences in the higher M.Wt range. Such differences could originate from the abolition or changes of restriction sites which might have arisen from genetic events such as insertion, deletion, inversion and transposition.

The present study confirms that PFGE is a powerful tool for molecular epidemiological studies at a level of precision not achievable by phage typing. The higher discriminatory power of PFGE over phage typing is evident especially in cases where different PFGE profiles were produced from isolates belonging to the same phage type. Determination of genetic relationships by PFGE and Dendron analysis clearly delineated the multiclonal nature of *S. typhi* isolates from countries around Asia. This supports the findings of Thong and colleagues [26] who demonstrated the genetic heterogeneity of *S. typhi* isolates from sporadic outbreaks and the genetic identity of isolates derived from common outbreaks in Malaysia.

In recent years, multilocus enzyme electrophoresis (MLEE) has served as the most common method for delineating the genetic structure of salmonella populations [27]. Reeves and colleagues [18] concluded that *S. typhi* was a single clone based on enzyme identity of 26 isolates from diverse geographical regions. However, in a separate study that investigated 334 *S. typhi* isolates, Selander and colleagues [28] established the presence of two major and five subclones. The study of total genomes of *S. typhi* isolates by PFGE has now provided an alternative method for studying the genetic relationships among epidemiologically important strains. The larger number of clones distinguished in the present study indicates a greater genetic heterogeneity among the *S. typhi* isolates studied and the higher discriminatory power of PFGE as compared to MLEE.

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