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Analysis of *Salmonella typhi* Isolates from Southeast Asia by Pulsed-Field Gel Electrophoresis

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Pulsed-field gel electrophoresis (PFGE) revealed that multiple genetic variants of *Salmonella typhi* are simultaneously present in Southeast Asia and are associated with sporadic cases of typhoid fever and occasional outbreaks. Comparative analysis of PFGE patterns also suggested that considerable genetic diversity exists among *S. typhi* strains and that some PFGE patterns are shared between isolates obtained from Malaysia, Indonesia, and Thailand, implying movement of these strains within these regions of Southeast Asia, where they are endemic.

Typhoid fever continues to pose an important public health challenge in many developing countries, with an annual incidence of 16 to 17 million cases and approximately 600,000 deaths. This threat is especially pronounced in the Southeast Asian region, with its rapid pace of economic development. As a consequence of economic growth, extensive, reciprocal movements of migrant workers are occurring between the neighboring countries of Malaysia, Thailand, and Indonesia, which has one of the highest incidences of typhoid fever in the world at more than 1,000 cases per 100,000 inhabitants. This points to the very real possibility of movement of *Salmonella typhi* strains among these countries. The problem of the movement of strains is made more urgent by the increasing incidence of antibiotic-resistant strains and the observation of more severe clinical disease among typhoid fever cases in Indonesia (6), which may be associated with more virulent strains. The potential for movement of *S. typhi* strains underpins the need for effective epidemiological surveillance as a basis for the development of rational control strategies. With regard to the molecular epidemiology of bacterial pathogens, we have witnessed an increasing interest in the development of molecular approaches which are reproducible and highly discriminatory for differentiating individual strains of bacterial pathogens. These approaches include multilocus enzyme electrophoresis, restriction endonuclease analysis, ribotyping, pulsed-field gel electrophoresis (PFGE), PCR-based profiling, and nucleotide sequence analysis (7). PFGE, in particular, has been widely used recently in molecular epidemiological investigations of infections caused by a large number of bacterial pathogens, including *S. typhi* (15). In a previous study, we used PFGE to analyze *S. typhi* isolates from sporadic cases and from outbreaks in Malaysia (15). It has been proposed recently that PFGE is able to differentiate between clonally related strains (one or two band differences) and strains which represent independent clones (differences in three or more bands) (8). We report here the use of PFGE in a comparative molecular characterization of *S. typhi* isolates from Malaysia, Thailand, and Indonesia. We show the considerable diversity and sharing of molecular types of *S. typhi* in Southeast Asia, implying extensive movement of strains among these three countries, where *S. typhi* is endemic.

S. typhi isolates were obtained from the University Hospital (120 isolates) and the Institute for Medical Research (60 isolates), Kuala Lumpur, Malaysia; Siriraj Hospital, Bangkok, Thailand (10 isolates); and the Cipto Mangunkusumo Hospital, Jakarta, and the Dr. Sutomo Hospital, Surabaya, Indonesia (50 isolates) (Table 1). All isolates were obtained from sporadic cases of typhoid fever within the same time period (1987 to 1994). The organisms were isolated, maintained, and identified by standard biochemical and serotyping methods (3). All isolates were tested for antibiotic susceptibility by standard disk diffusion procedures for measuring resistance (3) and were susceptible to ampicillin, chloramphenicol, kanamycin, streptomycin, co-trimoxazole, and tetracycline. Preparation of DNA for restriction endonuclease digestion and subsequent analysis by PFGE were done as described previously (15). *S. typhi* chromosomal DNA was digested with restriction endonucleases *Xba*I (5′-TCTAGA-3′), *Spe*I (5′-ACTAGT-3′), and *Avr*II (5′-CCTAGG-3′), and DNA fragments were separated by a contour-clamped homogeneous electric field gel electrophoresis method on a CHEF DR-II or CHEF DR-III system (Bio-Rad Laboratories, Richmond, Calif.). PFGE patterns were visually assessed, assigned arbitrary pattern types, and compared by calculating a similarity coefficient (F, proportion of shared fragments between two isolates) (4). The F value was calculated by using the following formula: $F = 2n_{xy}/(n_x + n_y)$, where n_x is the total number of DNA fragments from isolate x, n_y is the total number of DNA fragments from isolate y, and n_{xy} is the total number of DNA fragments that were identical in the two isolates. Isolates were considered to be genetically similar or identical if there was complete concordance of the DNA fragment profiles and were considered different, for pattern comparison only, if there was a difference of one or more DNA bands. With this method, an F value of 1.0 indicates identical patterns and an F value of 0 suggests complete dissimilarity. PFGE patterns were also analyzed by using a computer program for analysis of electrophoretic patterns

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TABLE 1. Numbers of isolates of *S. typhi* from Malaysia, Thailand, and Indonesia and numbers of PFGE patterns following digestion with *Xba*I, *Spe*I, and *Avr*II

Location (yrs of isolation)	No. of isolates tested	No. of PFGE patterns (range of *F* values)		
		*Xba*I	*Spe*I	*Avr*II
Malaysia (1987–1992)	60	48 (0.58–1.0)	48 (0.64–1.0)	48 (0.65–1.0)
Indonesia (1992, 1994)	50	30 (0.53–1.0)	30 (0.55–1.0)	30 (0.60–1.0)
Thailand (1987–1992)	10	9 (0.65–1.0)	9 (0.64–1.0)	9 (0.67–1.0)

(GelCompar; Applied Maths, Kortrijk, Belgium) (16) to generate similarity matrices and dendrograms. Dendrograms were generated by using a clustering approach based on the unweighted pair group arithmetic means method.

S. typhi isolates from Malaysia, Indonesia, and Thailand were analyzed following digestion with *Xba*I, *Spe*I, and *Avr*II. PFGE analysis of the various isolates produced patterns consisting of 6 (with *Avr*II) to 20 or 21 fragments ranging in size from 23 to 540 kbp (Fig. 1). Multiple PFGE patterns were detected among all of the isolates from all three countries after digestion with all three restriction endonucleases (Table 1). Significant diversity was also detected among these isolates as evidenced by the *F* values, which ranged from 0.53 to 1.0 (for *Xba*I), 0.55 to 1.0 (for *Spe*I), and 0.6 to 1.0 (for *Avr*II). Analysis of selected representative isolates from the three countries on the same gel (Fig. 1) and construction of a matrix of *F* values for these isolates showed that certain PFGE patterns that are either identical or very similar (*F*, 0.93 to 0.96) are shared by isolates from the three countries studied (Fig. 2A). Dendrogram analysis of some selected isolates also showed the close clustering of *S. typhi* isolates from Malaysia, Indonesia, and Thailand (Fig. 2B); at the 85% level of similarity, three clusters of isolates were evident, with two of the three clusters containing isolates from all three countries (Fig. 2B). At the same time, and on the basis of previously stated criteria for independent clones, many independent, clonally unrelated strains were detected in the three regions (Fig. 2A). The PFGE profiles generated by PFGE were both stable and reproducible when repeated analysis of these strains was performed.

Until the advent of molecular techniques, differentiation of individual *S. typhi* strains was problematic. Plasmid profiles are not very useful, as only a small proportion (<10%) of strains harbor plasmids (12). Vi phage typing is technically demanding, and analysis of envelope proteins detected only minor differences between strains (5). In recent years, the value of molecular methods for discriminating between strains of pathogenic bacteria in defined epidemiological settings has been well proven in numerous studies, including reports on *S. typhi* (1, 9, 15) and other pathogenic *Salmonella* spp. (2, 10). For example, rRNA gene restriction pattern (ribotyping) analysis was able to discriminate *S. typhi* strains (1, 9). In another study (15), we have reported that PFGE following digestion of *S. typhi* chromosomal DNA with *Xba*I, *Spe*I, and *Avr*II was able to differentiate isolates of *S. typhi*; it was observed that there was considerable diversity among *S. typhi* isolates associated with sporadic cases in Malaysia but that outbreak isolates were more clonal in nature. The present report extends the epidemiological scope of the original study to include the neighboring countries of Thailand and Indonesia. The extent of genetic diversity among *S. typhi* isolates associated with sporadic cases

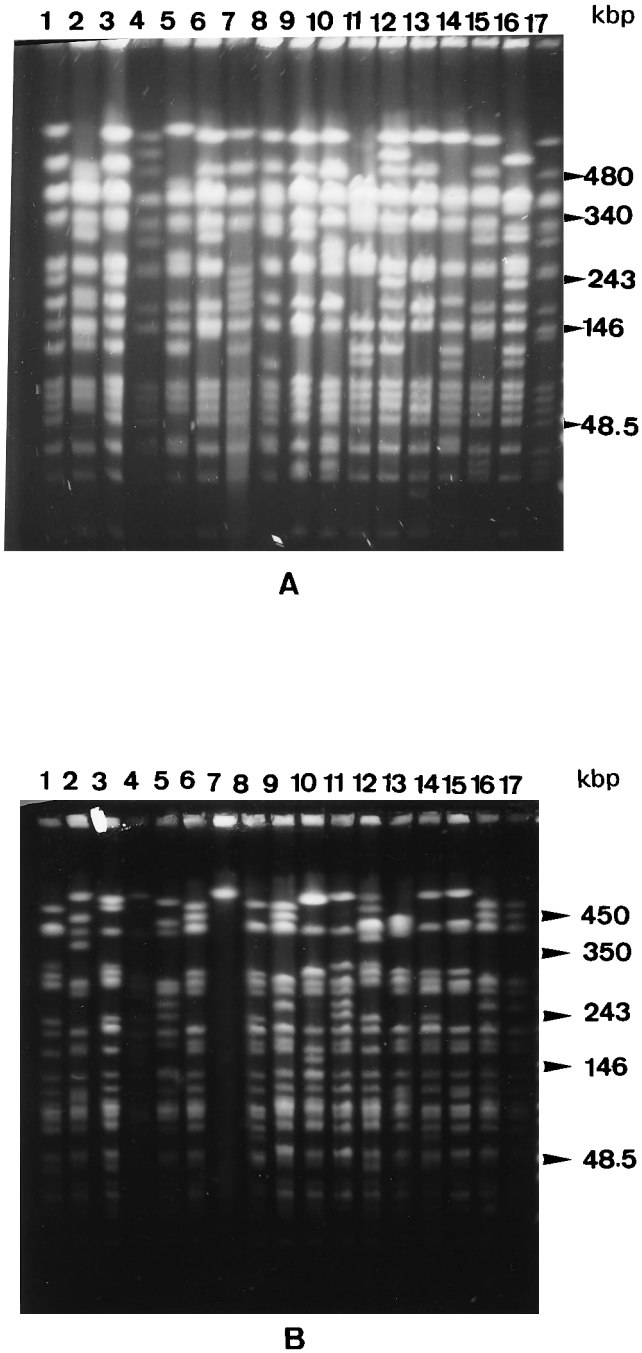

FIG. 1. PFGE patterns of representative *S. typhi* isolates from Malaysia, Thailand, and Indonesia after digestion of chromosomal DNA with *Xba*I (A) and *Spe*I (B). (A) Lanes: 1 to 3 and 5 to 8, Malaysian isolates; 4 and 10 to 13, Thai isolates; 9 and 14 to 17, Indonesian isolates. (B) Lanes: 1 to 3 and 7 to 9, Malaysian isolates; 4 to 5 and 11 to 15, Thai isolates; 6, 10, 16, and 17, Indonesian isolates; 7, undigested DNA. Positions of marker bands are indicated on the right.

of typhoid fever in Thailand and Indonesia, as assessed by PFGE patterns, is in close agreement with that reported previously for Malaysia (15). More importantly, the sharing of identical or closely related PFGE patterns among the isolates implies mobility and movement of the strains in Southeast Asia. This finding is perhaps not surprising in consideration of

A

M1	100														
M2	86.4	100													
In1	80.8	86.2	100												
M11	86.8	86.0	91.4	100											
T8	79.9	86.7	90.2	88.6	100										
In4	66.7	81.5	77.3	68.3	80.7	100									
M9	61.6	83.5	75.8	66.0	78.7	96.5	100								
In3	75.4	87.4	87.8	77.4	86.2	94.2	91.4	100							
M13	74.5	91.2	87.3	81.7	89.6	90.4	93.2	92.5	100						
T6	53.5	75.5	79.0	66.6	77.1	91.1	93.8	88.2	90.2	100					
In2	60.5	80.3	70.8	62.3	63.7	79.3	85.6	83.3	86.2	84.7	100				
T7	66.7	88.2	79.9	68.8	74.9	88.3	93.3	90.5	92.3	90.0	95.1	100			
M12	61.5	77.1	69.9	60.9	61.7	89.6	88.9	83.4	79.5	85.8	79.8	87.9	100		
M14	57.8	71.3	71.8	60.5	58.4	64.7	68.7	76.0	75.9	75.6	85.0	82.9	69.3	100	
M15	69.8	79.3	74.5	69.8	64.6	69.3	71.9	77.4	78.0	72.2	82.1	82.6	72.2	88.2	100
M16	67.5	72.7	68.0	68.7	62.6	56.3	55.9	67.8	66.8	60.7	64.5	83.6	58.9	79.1	80.0 100

B

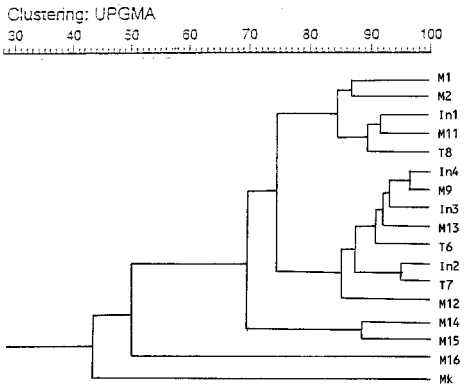

FIG. 2. (A) Matrix of *F* values for selected *S. typhi* isolates from Malaysia (M), Thailand (T), and Indonesia (In). The original *F* values have been multiplied by 100 to give similarity percentages. (B) Dendrogram showing the cluster analysis generated by the GelCompar program on the basis of *F* values and by using the unweighted pair group arithemtic means method. Mk, lambda concatemer PFGE marker.

the extensive movement of migrant workers and visitors among the three countries.

The present study also addressed the question of the clonality and genetic diversity of *S. typhi* strains circulating in areas where they are endemic, a subject which has received little attention. Previous studies have found that *S. typhi* strains from different geographic regions are virtually identical, which is compatible with the concept that *S. typhi* represents a single clone which has shown minimal genetic divergence in its spread to different parts of the world (13, 14). In a previous study using multilocus enzyme electrophoresis, Selander et al. (14) found a relatively low level of genotypic diversity among 334 isolates of *S. typhi* collected from numerous geographic sources; it was found that only two clones of *S. typhi* could be distinguished worldwide, with 82% of the total isolates belonging to one clone (Tp1) (14). In another study, envelope protein profiles detected only minor differences between strains (5). In contrast to these phenotypic approaches, genotypic techniques are believed to possess better reproducibility and discriminatory power (7). In a study of 69 *S. typhi* strains from Indonesia and Peru using envelope protein profiles, chromosomal restriction endonuclease digestion patterns, and immune response to envelope proteins, Franco et al. (5) concluded that there are genotypic and phenotypic differences among *S. typhi* strains. Ribotyping studies of *S. typhi* isolates from different parts of the world, including those from the United States, Sicily, and Malaysia (1, 9, 11), have also indicated considerable genetic diversity. This observation of genetic diversity has now been extended by use of PFGE, a method which scans for genetic variation over the entire bacterial chromosome. In relation to the significance of differences in PFGE profiles between individual strains, it has been proposed that strains with one or two band shifts caused by a single genetic event (e.g., a point mutation resulting in loss or gain of a restriction site, insertion, deletion, or chromosomal inversion) are considered to be clonally related (8). If a very limited number of band changes occur which are not due to a single genetic event, the strains are considered to be very closely related but distinct (8). Strains that differ in three or more bands are considered to be independent (8). On this basis, the PFGE findings described in the present study and previously (15) seem to argue against the previous concept that *S. typhi* isolates from different parts of the world belong to a single clone. They seem to indicate that multiple, independent clones of *S. typhi* exist in different parts of the world. It should be pointed out, however, that the possibility still exists that similar strains have a common ancestry. It is also important to emphasize that the phenotypic correlates of these genetic differences are unknown and the clinical significance of these genetic differences and their importance with regard to the virulence of individual strains remain to be evaluated. This remains as an important issue in research on typhoid fever in view of the observation that the reported clinical manifestations of typhoid fever differ markedly in different parts of the world (5, 6), including Southeast Asia.

The present report verifies the epidemiological usefulness of PFGE in characterizing and comparing strains of *S. typhi* in a region of the world where they are highly endemic. For molecular typing methods to be widely useful in comparative epidemiology, it is necessary to develop standardized protocols and also standardize the interpretation of results. In addition, results need to be validated in independent laboratories or in a reference laboratory. If this could be achieved, molecular typing of *S. typhi* strains, in conjunction with established typing methods, could eventually form the basis of an effective epidemiological surveillance system which would be invaluable in developing rational strategies to control this important disease and better understand its pathogenicity for humans.

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