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Molecular Analysis of Environmental and Human Isolates of *Salmonella typhi*

KWAI-LIN THONG,[1] ANA-MARIA CORDANO,[2] ROHANI M. YASSIN,[3] AND TIKKI PANG[4]*

Centre for Foundation Studies in Science[1] and Institute of Advanced Studies,[4] University of Malaya, 59100 Kuala Lumpur, and Bacteriology Division, Institute for Medical Research, Kuala Lumpur,[3] Malaysia, and Instituto de Salud Publica de Chile, Santiago, Chile[2]

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Molecular characterization of a total of 54 isolates of *Salmonella typhi* from Santiago, Chile, was performed by pulsed-field gel electrophoresis (PFGE) after digestion of chromosomal DNA with three restriction endonucleases: *Xba*I (5′-TCTAGA-3′), *Avr*II (5′-CCTAGG-3′), and *Spe*I (5′-ACTAGT-3′). Thirteen of the 54 isolates were obtained from environmental sources (sewage and river water), and the rest were isolates from clinical cases of typhoid fever. Considerable genetic diversity was detected among the human isolates obtained in 1994, as evidenced by the presence of 14 to 19 different PFGE patterns among 20 human isolates, with *F* (coefficient of similarity) values ranging from 0.69 to 1.0 (*Xba*I), 0.61 to 1.0 (*Avr*II), and 0.70 to 1.0 (*Spe*I). A total of eight phage types were detected among these 20 isolates, with 50% possessing the E1 or 46 phage type. There was no correlation between PFGE pattern and phage types. Similar diversity was seen among 21 isolates obtained in 1983, with 17 to 19 PFGE patterns detected and *F* values of 0.56 to 1.0 (*Xba*I), 0.55 to 1.0 (*Avr*II), and 0.67 to 1.0 (*Spe*I). Comparison of these two groups of human isolates obtained 11 years apart indicated that certain molecular types of *S. typhi* are shared and are able to persist for considerable periods. A similar degree of genetic diversity was also detected among the environmental isolates of *S. typhi*, for which 10 to 12 different PFGE patterns were detected among the 13 isolates analyzed, with *F* values ranging from 0.56 to 1.0 (*Xba*I), 0.52 to 1.0 (*Avr*II), and 0.69 to 1.0 (*Spe*I). Certain molecular types present among the environmental isolates of *S. typhi* were also found among the human isolates from the same time period, providing evidence for the epidemiological link between environmental reservoirs and human infection.

In many developing countries, typhoid fever remains an important public health problem, with 16.6 million cases and 600,000 deaths annually (14). In areas where typhoid fever is endemic, water from rivers or lakes which is used for public consumption and is sometimes contaminated by raw sewage is the main source of infection (9). It has been clearly demonstrated that the incidence of typhoid fever decreases dramatically with the provision of clean water through chlorination and filtration (9). Thus, in many developing countries where the use of raw river water remains widespread, this pathway of transmission remains an important factor in disease epidemiology. However, despite the clear importance of environmental sources of *Salmonella typhi*, little is known about the biochemical and molecular characteristics of such strains of *S. typhi* and the mechanisms of survival employed by this pathogen. Very few molecular studies have been carried out on these strains of *S. typhi* mainly because of the well-known difficulty in isolating this organism from environmental sources (3, 5, 15). Multiple resistance to antibiotics among *S. typhi* strains has become an important problem in recent times (14), and it is conceivable that transfer of antibiotic resistance can occur in aquatic environments. It is clear that a better definition of the molecular epidemiology of *S. typhi*, including analysis of strains obtained from environmental sources, would benefit greatly from the application of recently developed molecular typing techniques. We report here the results of molecular analysis by pulsed-field gel electrophoresis (PFGE) on environmental and human isolates of *S. typhi* from Santiago, Chile. PFGE analysis was undertaken to determine (i) whether the isolates were identical or different, (ii) whether environmental isolates were similar or identical to human isolates, and (iii) the extent of genetic diversity among the isolates.

Environmental and human isolates of *S. typhi* were used in this study. A total of 13 environmental isolates were obtained during the summer of 1983 (January to March) from sewage or water from the Mapocho River, Santiago, Chile, using Moore swabs as described previously (5). A total of 41 human isolates from blood were also obtained from sporadic cases of typhoid fever in Santiago, Chile; 21 isolates were obtained in 1983, and 20 were obtained during 1994. The organisms were isolated, maintained, and identified by standard methods (6). The isolates studied belonged to multiple phage types, and typing was performed according to standard procedures by the Salmonella Reference Centre at the Institute for Medical Research, Kuala Lumpur. As reported previously, most of the isolates belonged to phage types E1 and 46 (22). Repeated subculturing of isolates was avoided, and stocks of the primary isolates were maintained at −70°C. All *S. typhi* isolates tested were sensitive to ampicillin, amikacin, chloramphenicol, kanamycin, carbenicillin, cephalothin, cefamandole, gentamicin, neomycin, tetracycline, trimethoprim, streptomycin, spectinomycin, sulfonamides, nitrofurans, and nalidixic acid, as determined by standard disk diffusion procedures to measure resistance (National Committee for Clinical Laboratory Standards guidelines) (12). None of the isolates studied contained any plasmids, as determined by a standard alkaline lysis procedure and by PFGE of undigested DNA (see below).

DNA for PFGE analysis was prepared by a modification of the method of Smith et al. (19) as described previously (20).

* Corresponding author. Mailing address: Institute of Advanced Studies, University of Malaya, 59100 Kuala Lumpur, Malaysia. Phone: 60 3 7594437. Fax: 60 3 7568940. Electronic mail address: tikkip@pc.jaring.my.

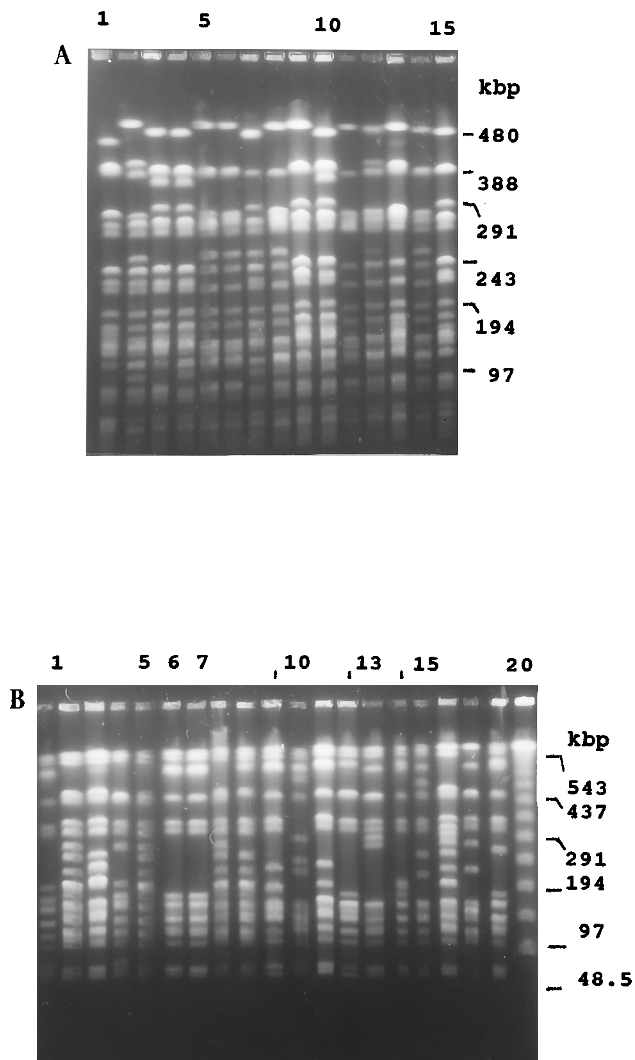

FIG. 1. PFGE patterns of environmental and human isolates of *S. typhi* following digestion with *Xba*I (A) and *Spe*I (B). (A) Lanes: 1 and 2, human (1983); 3 to 15, environmental (1983). (B) Lanes: 1 to 9, environmental (1983); 10 to 18, human (1983); 19, *S. typhi* ATCC 19430; 20, lambda concatemers (markers).

Selection of restriction enzymes was based on the recognition site of the enzyme and the G+C content of 50 to 54% previously reported for *Salmonella* spp. The following restriction endonucleases were used: *Xba*I (5′-TCTAGA-3′), *Avr*II (5′-CCTAGG-3′), and *Spe*I (5′-ACTAGT-3′) (New England Biolabs, Beverly, Mass.; Stratagene Co., La Jolla, Calif.). PFGE of inserts was performed by the contour-clamped homogeneous electric field (CHEF) method on a CHEF DR-II or DR-III system (Bio-Rad Laboratories, Richmond, Calif.) in gels of 1% agarose in 0.5× TBE buffer (0.1 M Tris, 0.1 M boric acid, 0.2 mM EDTA) for 28 to 30 h at 200 V at a temperature of 14°C, with ramped pulse times varying according to the enzymes used (ranging from 2 to 50 s). Gels were stained with ethidium bromide and photographed with a UV transilluminator (Spectroline; 302 nm). The DNA size standards used were a lambda ladder consisting of concatemers starting at 48.5 kbp and increasing to approximately 1,000 kbp (Bio-Rad Laboratories) and a mid-range II PFG Marker (size range, 24 to 291 kbp) (New England Biolabs). Size determination of DNA fragments was carried out by measuring migration distances of individual bands and referring these to a standard curve which plotted migration distances against log molecular weight of the DNA size standards. For each strain, with various restriction endonucleases, 15 to 24 bands were normally seen, and all visible bands were included in determining PFGE patterns. The existence of doublets (comigrating bands) was also taken into account by referring to band intensity. Several electrophoretic runs with ramped pulse times varying between 2 and 50 s were performed to obtain optimal times in which individual bands were clearly separated. DNA fragment patterns were visually assessed, and distinct patterns were assigned an arbitrary PFGE pattern. Isolates were considered genetically similar or identical if there was complete concordance of DNA fragment profiles and considered different if there was a difference of one or more DNA bands. Patterns generated by PFGE for various isolates were compared, and the similarity of fragment length patterns between two strains is scored by the Dice coefficient, also known as a coefficient of similarity (7). This coefficient, F, expresses the proportion of shared DNA fragments in two isolates and was calculated by using the 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 from isolate Y, and n_{xy} is the number of fragments identical in the two isolates. An F value of 1.0 indicates that the two isolates have identical PFGE patterns.

PFGE analysis of the various *S. typhi* isolates was carried out following digestion of chromosomal DNA with three restriction endonucleases: *Xba*I (5′-TCTAGA-3′), *Avr*II (5′-CCTAGG-3′), and *Spe*I (5′-ACTAGT-3′). Stable and reproducible PFGE patterns comprising 15 to 24 DNA fragments were regularly obtained with the three enzymes, and representative results with two of these enzymes are shown in Fig. 1. Analysis of these PFGE patterns showed that between 14 and 19 different PFGE patterns were present among the 20 human isolates obtained in 1994, with F (coefficient of similarity) values ranging from 0.69 to 1.0 (*Xba*I), 0.61 to 1.0 (*Avr*II), and 0.70 to 1.0 (*Spe*I) (Table 1). Among these 20 isolates from 1994, 10 (50%) were phage types E1 and 46, with six other phage types distributed among the remainder. There was no correlation between the phage types and PFGE patterns of the isolates. Similar diversity was seen among 21 isolates obtained in 1983, with 17 to 19 PFGE patterns detected and F values ranging from 0.56 to 1.0 (*Xba*I), 0.55 to 1.0 (*Avr*II), and 0.67 to 1.0 (*Spe*I) (Table 1). Three *Xba*I, four *Avr*II, and two *Spe*I PFGE patterns were present or shared between isolates in the groups obtained in 1994 and 1983 (F = 1.0).

Analysis of 13 environmental isolates of *S. typhi* from sewage and from the river Mapocho, also obtained in 1983, indicated a similar extent of diversity (Fig. 1). A total of 11 or 12 different PFGE patterns were detected among the 13 isolates analyzed, with F values ranging from 0.56 to 1.0 (*Xba*I), 0.52 to 1.0 (*Avr*II), and 0.69 to 1.0 (*Spe*I) (Table 1). Based on comparison of PFGE patterns, two *Spe*I patterns and two *Avr*II patterns were shared between these environmental isolates and human

TABLE 1. F values and PFGE patterns for *S. typhi* isolates from Santiago, Chile

No. of isolates	Source and yr of isolation	F value range (no. of PFGE patterns)		
		*Xba*I	*Spe*I	*Avr*II
20	Human, 1994	0.69–1.0 (19)	0.70–1.0 (14)	0.61–1.0 (19)
21	Human, 1983	0.56–1.0 (19)	0.67–1.0 (18)	0.55–1.0 (17)
13	Environmental, 1983	0.56–1.0 (12)	0.69–1.0 (11)	0.52–1.0 (10)

isolates obtained in the same year (F = 1.0) (Fig. 1B, lanes 6, 7, and 13). With the enzyme *Xba*I, four very similar PFGE patterns, differing by only a single band (F = 0.97), were shared between the human and environmental isolates. Identical, shared PFGE patterns were also observed between the environmental isolates of 1983 and the human isolates obtained in 1994. None of the isolates studied contained any plasmids, as determined by a standard alkaline lysis procedure and by PFGE of undigested DNA.

Typhoid fever continues to be a major public health problem in developing countries as a result of many interrelated factors, including increased urbanization, inadequate supplies of clean water, antibiotic resistance, variable efficacy of vaccine preparations, and increased regional movement of large numbers of migrant workers. Despite the clear need for more effective epidemiological surveillance based on discriminative markers, the differentiation of individual *S. typhi* strains has been problematical in the past. Plasmid profiles are not very useful, as only a small proportion (<10%) of strains harbor plasmids (16). Vi phage typing has been of some value but is technically difficult and can be performed only by reference laboratories. In addition, a significant proportion of strains (22%) are not typeable, and some are Vi negative (4). Analysis of envelope protein profiles of *S. typhi* strains isolated in Peru and Indonesia detected only minor differences between strains (8). Recently, molecular analysis has provided important new insights into the molecular epidemiology and genetic diversity of *S. typhi*. Earlier studies utilizing the techniques of multilocus enzyme electrophoresis concluded that *S. typhi* represents a single clone that has shown minimal intraspecies divergence in its spread to different parts of the world (17, 18). More recently, the development of PFGE, by which large DNA fragments are separated after digestion of chromosomal DNA with infrequently cutting restriction endonucleases, has provided a new approach to the molecular typing and differentiation of individual strains of a variety of bacterial pathogens (2, 10). By this approach, studies of *S. typhi* isolates from different parts of Southeast Asia suggested that the extent of genetic diversity among strains of *S. typhi* is actually greater than was thought previously (11, 20, 21). The considerable genetic diversity among *S. typhi* strains has also been shown by ribotyping (1, 13).

The present study has extended this observation of significant diversity to human isolates of *S. typhi* from South America, an important typhoid fever-endemic area. It is clear from the data obtained that considerable heterogeneity exists at the DNA level among *S. typhi* isolates from Santiago, Chile. In overall terms, the South American strains (F = 0.55 to 1.0) appear to possess the same amount of diversity as the Southeast Asian strains (F = 0.53 to 1.0) (21). The present study also confirms the discriminatory power of PFGE, in that 14 to 19 PFGE patterns were detected, compared with eight phage types, among the 20 human isolates obtained in 1994. The results also suggest that multiple clones of *S. typhi* are endemic to Chile and coexist simultaneously to cause sporadic cases of typhoid fever among the population throughout the year and that no single clone predominates. Comparative analysis of isolates obtained 11 years apart also indicates that certain genotypes of *S. typhi* are stably maintained and persist over this considerable time period. The results of the present study with *S. typhi* strains from South America, together with those of previous studies (20, 21), provide further support for the hypothesis that multiple clones of *S. typhi* coexist independently and simultaneously in regions of the world where it is endemic, in contrast to the previous notion that all *S. typhi* strains belong to a single clone worldwide (17, 18). This conclusion is in agreement with the observation that recent studies of microbial populations have shown substantial genetic diversity within species which suggest that isolates are distributed among many genetically diverging lineages (2). It has been suggested that this evolutionary divergence is a reflection of the accumulation of random, nonlethal point mutations, including single-base substitutions and deletions, deletion of single genes, or even substitutions of DNA from other microbial species (2).

With a water-borne disease such as typhoid fever, the environmental reservoirs of *S. typhi* and the mode of survival of this organism in the environment are clearly important in disease epidemiology and transmission. We report here the molecular characterization of strains of *S. typhi* isolated from environmental sources and their relationship with human isolates. In contrast to studies involving other water-borne pathogens, which are relatively easy to isolate from water samples, studies with *S. typhi* from the environment have been hampered in the past by the well-documented difficulty in obtaining environmental isolates of *S. typhi* (3, 15), perhaps because of fastidious nutritional requirements, a dormant, viable, but nonculturable state, or the abundant presence of phages lytic for *S. typhi* (5). The environmental isolates of *S. typhi* analyzed in the present study were obtained from the river Mapocho in Santiago, Chile, known to be heavily polluted with sewage discharge (5, 22). The results in the present study suggest, not surprisingly, that similar genetic diversity exists among *S. typhi* from environmental sources as among the human isolates. Also, most of the molecular types detected among these environmental isolates of *S. typhi* were also present among the human isolates obtained during the same time period in the same locality. This observation provides convincing evidence, at the molecular level, of the epidemiological link between water supplies contaminated by sewage and human infection with *S. typhi.*

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